Přírodovědecká fakulta Univerzity Karlovy v Praze
Katedra fyziologie živočichů - neurobiologie

Bc. Martin Šubrt

Long-term functional consequences of early clonazepam exposure in immature rats

Diplomová práce
Praha 2009

Školitelka Doc. PharmDr. Hana Kubová, DrSc.

Prohlášení

Prohlašuji, že jsem svou diplomovou práci vypracoval samostatně s pomocí odborného školitele a použil jsem pouze podklady uvedené v seznamu literatury. Na provedení studie a získání výsledků se významně podílela RNDr. Anna Mikulecká, PhD.

Poděkování

Rád bych poděkoval paní Doc. PharmDr. Haně Kubové, DrSc. vedoucí mé diplomové práce za trpělivé vedení, cenné rady a ochotu. Dále patří velký dík RNDr. Anně Mikulecké PhD., která se podílela na provedení studie významnou měrou a poskytla cenné vědomosti o behavioralních studiích. Poděkování si zaslouží i všichni další členové oddělení vývojové epileptologie, za ochotu a vstřícnost, se kterou jsem se u nich během práce setkal. V neposlední řadě bych rád poděkoval svým rodičům za podporu a zázemí, které mi poskytovali během celého vysokoškolského studia.

Abstrakt

Vzhledem k širokému spektru účinnosti představují benzodiazepiny (BZs) jedny z nejčastěji předepisovaných léčiv. V určitých indikacích jsou podávány i během těhotenství a novorozencům či kojencům. Klinické studie však naznačují, že podávání těchto látek v časných stadiích vývoje může dlouhodobě ovlivnit chování jedince. V klinické praxi je těžké oddělit následky časného podávání BZs od změn spojených s vlastním onemocněním. Jednou z možností, jak stanovit rizika podávání těchto látek pro vyvíjející se mozek je použití relevantních experimentálních modelů. Úkolem předkládané studie bylo zhodnotit rizika podávání clonazepamu (CZP) pro další vývoj funkčních schopností jedince. Mláďatům potkana byl podáván CZP v terapeuticky relevantních dávkách (0.1 – 1 mg/kg/den) v průběhu pěti dní (od postnatálního dne /P/ 7 do P11) Zvířata byla opakovaně testována s použitím baterie behaviorálních testů hodnotících kognitivní funkce, motivaci a emocionalitu od P12 až P90.

Výsledky ukazují, že podávání CZP v testovaných dávkách ve fázi vývoje odpovídající stadiu lidského nedonošence až novorozence nevede k porušení paměti a schopnosti učení s výjimkou dlouhodobé habituace. Dochází však ke zvýšení lokomoční activity a snížení motivace. V období adolescence byla u zvířat, kterým byl CZP podáván, nalezena změna sociálního chování.

Abstract

Benzodiazepines (BZs) are widely prescribed because of broad spectrum of therapeutic effects. Under specific conditions they can be used during the pregnancy and early postnatally in humans. However, clinical studies suggest their possible behavioral teratogenicity even though it is difficult to separate consequences of early BZs exposure from underlying pathologies in clinical practice. Therefore experimental studies can help us to determine risks of early BZs treatment for later development.

Present study was designed to assess functional consequences of early exposure to clonazepam (CZP). Immature rats were exposed to therapeutically relevant doses of CZP (0.1 – 1 mg/kg/day). CZP was administered for five consecutive day starting at postnatal (P) day 7. Behavioral tests started 24 hours after the last CZP injection (i.e. at P12) and continued up to the age of 4 months. Battery of behavioral tests evaluating cognitive functions, motivation and emotionality was used in this study.

Present data show that early CZP exposure does not affect memory and learning abilities, but it leads to the increase of locomotor activity, decreased motivation and impairment of long term habituation. None of these effects was clearly dose-dependent. Importantly, early CZP-exposure changed social behavior (social interactions related to play) in adolescent rats in dose-dependent manner.

In conclusion, even relatively short exposure of immature rats to therapeutically relevant doses of CZP results in long-lasting functional deficits later in the life.

Contents

List of abbreviations

BZ – benzodiazepine

BZs – benzodiazepines

CZP – clonazepam

DGCs – dentate granule cells

EEG – electroencaphalogram

EPM – elevated plus maze

GABA – gamma-aminobutyric acid

KCCs – K^+-Cl^- co-transporters

NKCCs – Na^+-K^+-$2Cl^-$ co-transporters

mRNA – messanger ribonucleic acid

MWM – Morris water maze

P – postnatal

PD – postnatal day

PTZ – pentylmethylenetetrazole

1. Introduction

Changes of some behavioral parameters represent serious risk of drug administration in human infants and children. New drugs are developed in experimental models which use only adult animals and even though they are determined for all age groups, tests in developing animals are not requested. In mammals, brain development still continues after birth and thus developing brain is very prone to effects of exogenous agents. The risk of drug-induced functional or developmental alterations is substantially higher when long-lasting treatment is necessary and highly effective drugs, interacting with specific targets in the brain are used. Antiepileptic drugs represent typical example of this drug category.

2. Overview of literature

2.1 Benzodiazepines and their usage

Benzodiazepines (BZs) (Fig.1) were introduced into clinical practice in 1960. Chlordiazepoxide (trade name Librium) was first released BZ, followed by introduction of famous diazepam (Valium). Since that time, over 50 BZs have been introduced into clinical practice. They have become one of the most frequently prescribed psychotrophic drugs. In 1973, at the period of their greatest popularity, over 87 million BZ prescriptions were filled throughout the world. Although the rate of BZs usage is declining, BZs still remain frequently prescribed psychotropic medication due to their rapid effect onset, minimal side effects, low toxicity, low risk of overdose and large therapeutic index. BZs are prescribed for their anxiolytic, hypnotic, sedative, muscle-relaxant and anticonvulsant properties. According to their elimination half-life BZs are categorized as either short-, intermediate- or long-acting (Buffet-Jerrot and Stewart, 2002; Haefely, 1988).

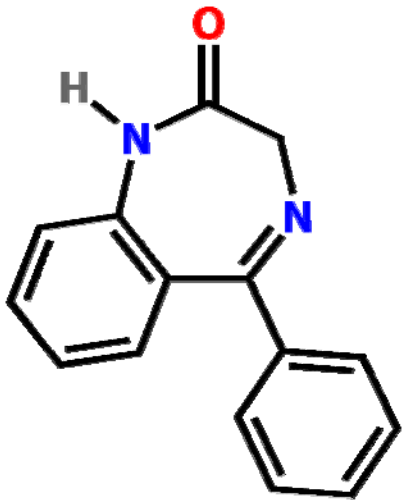

Fig.1 Core structure of benzodiazepines

BZs, in particular diazepam, have been reported to be among the most widely prescribed drugs in pregnancy, with 30 to 40% of all pregnant women being given anxiolytic drugs at some stage of pregnancy (McElthatton, 1994). They can be use to treat depression and in manic episodes to control agitation (Ebhard-Gran et al., 2005). BZs has been also shown to be effective in the managing preeclampsia or eclampsia, which is an acute and life-threatening complication in pregnancy (Iqbal et al., 2002), or as an supportive therapy before Caesarean delivery (Collis et al., 2002). About 0.5% of all pregnant women suffer from epilepsy and

treatment cannot be disconnected during pregnancy or nursing. Physiological, hormonal and pharmacokinetic changes in pregnancy may escalate seizure activity and BZs can be use to attenuate possible risks of seizures for developing fetus (Nulman et al., 1999).

BZs belong among the most potent anticonvulsant drugs and they are used in newborns, infants (even premature) and children suffering from epilepsy. Epidemiological studies suggest that 0.8 - 1% of the world population has epilepsy. The occurrence of epilepsy syndromes in children is significantly higher (~4%) than in adults. Many of these syndromes start to manifest during early infancy or childhood and they can be potentially dangerous for subsequent psychomotor development of children. BZs are at least partially effective in the treatment of atonic seizures, myoclonic seizures, atypical absence seizures, tonic seizures, and infantile spasms – seizures, which are among the most difficult to control in children. Thus, BZs play major role in the managament or seizure suppression in children (Browne and Penry, 1973; Farrell, 1986; Riss et al., 2008).

2.1.1 Adverse effects of benzodiapine use

BZs have been shown to have several adverse effects as described in this chapter. However, it has to be emphasized that these effects may significantly differ due to used type of BZ, dose and duration of treatment.

Until the 1970s, it was presumed that BZs did not significantly affect cognitive functions in humans. Later studies and longer experiences however demonstrated that BZs may alter human cognition. In adults, acute BZ administration has been known to produce sedation, drowsiness, psychomotor slowing, anterograde amnesia, and difficulties in learning (Buffet-Jerrot and Stewart, 2002; Stewart, 2005). Chronic treatment may lead to impaired visiuospatial and visuomotor abilities, decreased IQ, motor coordination, psychomotor speed, speed of information processes, verbal learning, memory and concentration. Conversely, there is evidence that individuals are likely to develop some tolerance to above mentioned adverse effects of BZs when administered for several weeks (Otto et al., 2005; Stewart, 2005).

Drowsiness represents the most common side effects of BZs administration and it is reported in 10-40% of patients. The second most common side effect of chronic benzodiazepine administration is incoordination, or ataxia, which develop in 5-20% of patient. Other common side effects include feeling of dizziness, agitation or irritability, and hypotonia. In addition, BZ treatment may result in weight loss (Browne and Penry, 1973).

Long-term use of BZs is connected with development of therapeutic tolerance, which could be manifested by either loss of efficacy or a need to escalate the dose to maintain benefit. Tolerance can be observed for all BZs effects. In the epilepsy treatment, BZs are generally considered unsuitable for long-term control of epilepsy because of possible development of anticonvulsant tolerance which was reported mainly in animal models (Riss et al., 2008), however they are still successfully used to treat some age-related epileptic syndromes or epilepsies as well as in epileptic emergencies (status epilepticus). In anxiety treatment, clinical data are somehow controversial. Some reports do not show development of tolerance whereas others demonstrate development of tolerance to anxiolytic effects (Stevens and Pollack, 2005).

Dependence develops in almost one-third of patients treated with BZs for more than four weeks and it is characterized mainly by a withdrawal syndrome with cessation of treatment or reduction of dose and it represents one of the most serious adverse effects of BZs (Marriott and Tyrer, 1993). Intensity of withdrawal symptoms ranges from mild (e.g. insomnia, gastric problems and anxiety) to severe (e.g. seizures and psychosis). Severity of withdrawal symptoms is related to used doses of BZs, duration of treatment, and also to history of drug abuse or personal pathology. To prevent withdrawal syndrome gradual tapering of BZs doses is recommended (O'Brain, 2005 ; Riss et al., 2008).

When are given to children BZs may cause the same adverse effects observed in adults like memory impairment, psychomotor speed decrease, motor coordination and sustained attention alterations (Werry and Aman, 1998).

BZs have been considered as the teratogenic substances, because they cross the placentar barrier. In the early 70's, studies reported that first trimester exposure to BZs in utero resulted in the birth of some infants with facial clefts, cardiac malformation, and other multiple malformations, but no syndrome or defects. However, data from later studies provide no clear evidence of a significant increase either incidence of malformation or of any particular type of defect (McElhatton, 1994). As a general rule, exposure to any type of BZ during the first three months of pregnancy should be avoided, because the fetus is the most vulnerable to toxic effects of drugs during this period of active neurogenenesis (Iqbal et al., 2002). Exposure to BZs during the late third trimester seems to be associated with risks for the fetus/neonate. Some, but not all infants exposed to BZs at this period of prenatal development, exhibit either floppy infant syndrome, or marked neonatal withdrawal symptomes. Symptoms vary from mild to sedation, hypotonia, and reluctance to suck, to apnoeic spells, cyanosis, and impared metabolic responses to cold stress. These symptoms can

persist for periods from hours to months after birth (Ebhard-Gran et al., 2005; McElhatton, 1994). BZs also accumulate in breast milk and may cause drowsiness of suckling infants (Kaneko, 1991).

2.2 Pharmacology of Benzodiazepines

2.2.1 GABAergic neurotransmission

Effects of BZs are mediated through their interaction with benzodiazepine receptor binding site that acts at synapses in which gamma-aminobutyric acid (GABA) is used as transmitter (Haefely et al., 1985).

GABA is the main inhibitory neurotransmitter in the brain and it plays an important role in regulating neuronal excitability throughout the nervous system (Fig.2). It is synthesized in the terminals of GABAergic neurons from glutamate by its decarboxylation catalysed by glutamate decarboxylase and stored in the synaptic vesicles of the nerve terminal. When the nerve impulse reaches the nerve terminal, GABA is released into the synaptic cleft. Afterwards, high affinity GABA transporters located in the presynaptic GABAergic neurons as well as surrounding astrocytes uptake and thus inactivate GABA. Subsequently, GABA may be metabolized by GABA-transaminase. In neurons, GABA seems to be preferentially recycled and reused in neurotransmission (Petroff, 2002; Madsen et al., 2007).

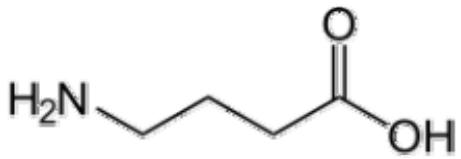

Fig.2 Molecular structure of gamma-aminobutyric acid

GABA released from GABA neurons into the synaptic cleft, interacts with the GABA receptors. There exist at least three different kinds of GABA receptors – $GABA_A$, $GABA_B$ and $GABA_C$

2.2.2 GABA receptors

$GABA_A$ receptor is a pentameric anion-selective channel (Fig.3) composed of seven subunit families comprising at least 18 subunits (Fig.4). Muscimol is the selective agonist and bicuculline is the selective antagonist of $GABA_A$ receptors. In mature brain, the binding of GABA causes conformational changes of receptor which increases the chloride influx and causes hyperpolarization of the postsynaptic membrane (Möhler, 2007; Sieghart, 1992).

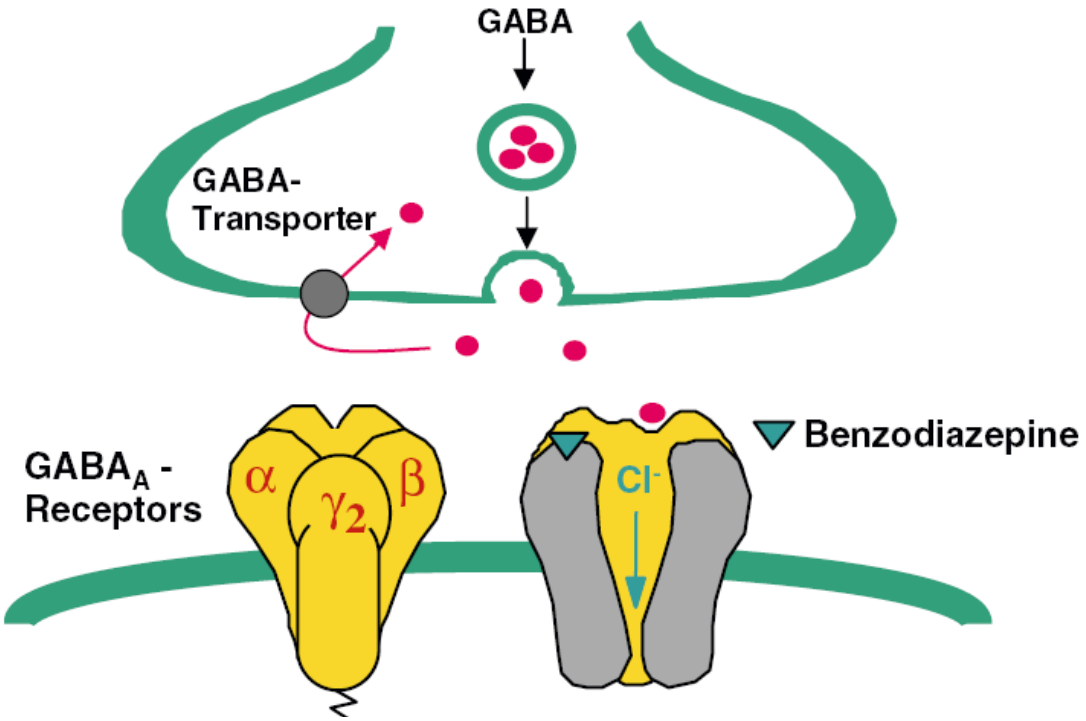

Fig.3 Scheme of GABAergic synapse depicting major elements of signal transduction (taken from Möhler 2007)

α 1-6
β 1-3
γ 1-3
δ 1
ε 1
θ 1
ρ 1-3

Fig.4 $GABA_A$ subunit repertoire (adapted from Möhler 2007)

There are several binding sites located at the subunits of the $GABA_A$ receptor complex. The binding site for GABA is located at the interface α and β subunits. The benzodiazepine binding site represents another well described modulatory site at GABA receptor; it is localized at the interface α and γ2 subunits (Fig.5). Once bound to the BZ binding site, BZs act as positive allosteric modulators and lock the $GABA_A$ receptor into a conformation where the neurotransmitter GABA has much higher affinity. This leads to increase of frequency of channel opening and consequently to the hyperpolarization of the membrane (Sigel, 2002). Other binding and modulatory sites were found for the barbiturates, neurosteroids, picrotoxins and some metal divalent cations (Sieghart, 1992).

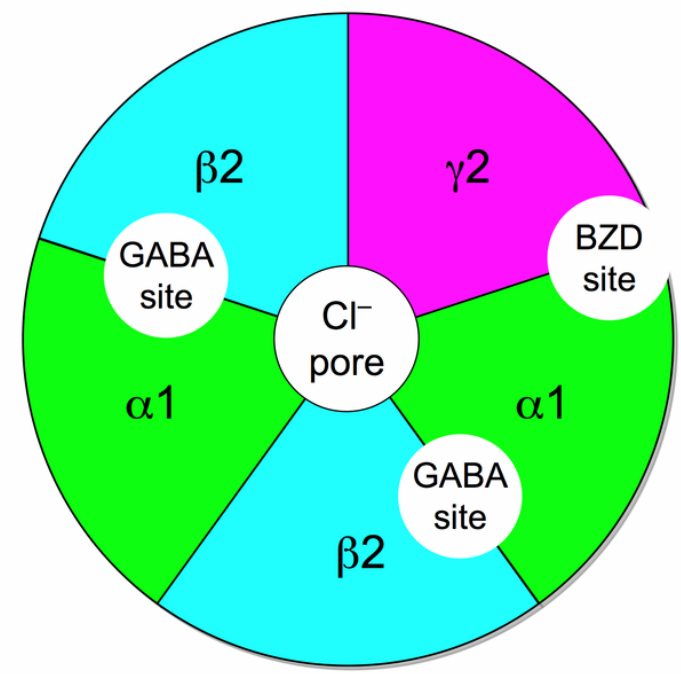

Fig.5 Subunit arrangement of $GABA_A$ receptor subunits. G – GABA binding site, B – BZs binding site

$GABA_B$ receptor, discovered in 1981, is located either at the presynaptic or postsynaptic membrane and is indirectly coupled via G-proteins to potassium channels. When potassium channel opens, the neuronal mebrane becomes hyperpolarized. Baclofen is the selective agonist and both saclofen and phaclofen represent selective antagonists at these bicuculline-insensitive receptors (Bowery, 2006).

In early 1990s, the **$GABA_C$ receptor** has been discovered. $GABA_C$ receptor acts as ligand-gated Cl^- channel which is not blocked by bicuculline and is resistant to the modulation effect of benzodiazepines, barbiturates or neurosteroids (Johnston, 1996).

2.2.3 $GABA_A$ receptor subtypes

$GABA_A$ receptor functions (channel kinetics, affinity for GABA, rate of desensitation etc.) and pharmacological sensitivity is determined by subunit composition (Möhler, 2006). Although there are many possible subunit combinations, most receptors appear to be formed by α, β and γ subunits in a 2:2:1 stoichiometry (Derry et al., 2004). Receptors can be distinguished by their in/sensitivity to diazepam.

Receptors containing the subunits α1, α2, α3 or α5 in combination with any of the β subunits and the γ2 subunit are most prevalent in the brain (85-95%). These receptors are sensitive to BZ modulation. The major receptor subtype is assembled from the subunits α1β2γ2. Only a few brain regions lack this receptor (e.g. reticular nucleus of thalamus or granular cell layer of the olfactory bulb) (Möhler, 2006, 2007; Pirker, 2000).

Receptors containing the α2 or α3 subunits are markedly less abundant. They are highly expressed in areas where the α1 subunit is absent or at low levels. The α2 and α3 subunits are frequently co-expressed with β3 and γ2 subunits, which are particulary noticeable in hippocampapal pyramidal neurons and in cholinergic neurons of the basal forebrain. α2- and α3-receptors differ from α1β2γ2 receptor by having a substantially lower displacing potency for various ligands (Möhler, 2006, 2007; Pirker, 2000).

Receptors containing the α5 are of minor abudance in the brain but they are expressed to a significant extent in the hippocampus, where they comprise 15-20% of the diazepam-sensitive $GABA_A$ receptor population. This population is mainly co-assembled with β3 and γ2 subunits. These receptors are pharmocologically differentiated from α1β2γ2, α2β2γ2 and α3β2γ2 receptors by lower affinity to certain substances (Möhler, 2006, 2007; Pirker, 2000).

$GABA_A$ receptors containing α4 and α6 subunits are characterised by insensitivity to clinically used BZs such as diazepam, flunitrazepam, clonazepam, and zolpidem and by low abudance in the brain. Receptors with α4 subunit are generally expressed at very low abudance but more prominently in thalamus and dentate gyrus. Receptors containing α6 subunit are found in the granular cell layer of the cerebellum. Both receptor populations are structurally very heterogenous. The δ subunit is frequently co-assembled with both α4 and α6 subunits and these receptors are characterised by high affinity for GABA and slow desensitation kinetics. Receptors containing δ subunit are located exclusively at extrasynaptic sites in dentate gyrus and cerebellum (Möhler, 2006, 2007).

2.2.4 Changes in GABAergic system and expression of $GABA_A$ receptor subunits during development

As mentioned before, GABA is the major inhibitor neurotransmitter in the brain and activation of GABA receptors results in the hyperpolarisation of adult neuronal membrane. Using hippocampal slices from newborn animals up to two weeks of age it was demonstrated that the activation of GABA synapses in young neurons produces a depolarization instead of characteristic hyperpolarization (Ben-Ari et al., 1989). Afterwards, in rodents, several observations confirmed previous discovery that the activation of $GABA_A$ receptors produces a depolarization and thereby increased concentration of intracellular calcium in immature but not adult neurons. Depolarization was observed in a wide range of brain structures, including hippocampus and neocortex (Barker et al., 1998).

This phenomena is caused by higher intracellular concentration of chloride $[Cl^-]_i$ (Fig.6) in immature neurons compared to adult neurons by 20-40 mM. This difference is sufficient to shift the actions of GABA from inhibition to excitation. In contrast to immature neurons, reversal potential to transmembrane chloride concentration is below the resting membrane potential in adult neurons. In the immature neurons, reversal potential to transmembrane chloride concentration is above the resting membrane potential and whenever $GABA_A$ receptors are open chloride anions leak out the neurons and membrane becomes depolarized and closer to the threshold for action potential (Ben-Ari, 2002).

The intracellular accumulation of chloride in immature neurons can be generated either by the early expression of an Na^+-K^+-$2Cl^-$ co-transporters (NKCCs) or by the delayed expression of K^+-Cl^- co-transporters (KCCs). The key feature of NKCCs is the transport chloride into the cell, whereas KCCs are characterized by transporting chloride out of the cell (Ben-Ari, 2002).

It seems that KCC2 is seminal for the switch from GABA-mediated excitation to inhibition. Increase of KCC2 mRNA expression correlated with the switch of GABA actions (Rivera et al., 1999). Blockade of $GABA_A$ receptors prevented from the switch – the KCC2 transporter was not expressed and GABA continued exerting depolarizing action. Thus, the KCC2 signal provides a feedback of $GABA_A$ receptors in which GABA itself promotes the shift from excitation to inhibition mediated by calcium signals. Those signals ultimately lead to the expression of a tranporter and consequently to reduction in $[Cl^-]_i$ (Ganguly et al., 2001).

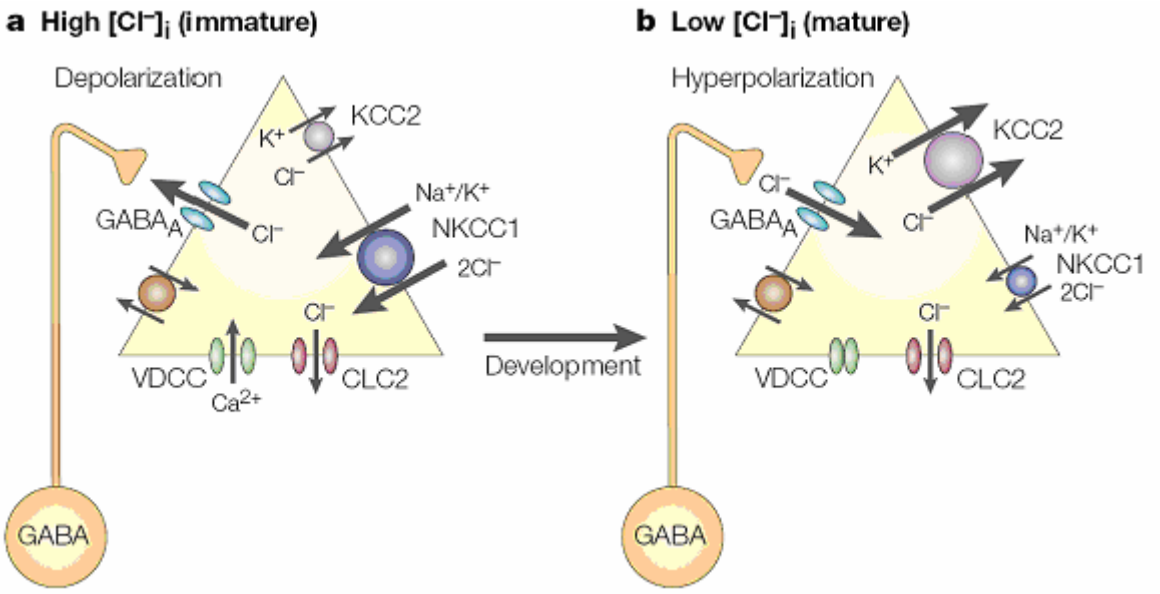

Fig.6 Determination of developmental changes $[Cl^-]_i$ in by early expression of NKCC1 and late expression KCC2. **a)** NKCC1 expression predominates in immature neurons, in which is $[Cl^-]_i$ relatively higher **b)** KCC2 expression predominates in mature neurons and $[Cl^-]_i$ is lower. Activation of $GABA_A$ receptor generates efflux of chloride (excitation) in immature neurons and influx (inhibition) in adult neurons. Abcissae: VDCC – voltage-dependent calcium channel, CLC2 – voltage-gated chloride channel 2 (taken from Ben-Ari 2002).

In addition, GABA-ergic neurons are present in proliferative, migratory and differentiation zones during embryonic development and GABA has been shown to positively stimulate several essential development functions, including neuronal migration, cell division and neuritic growth (Owens and Kriegstein, 2002).

Along with functional switch of GABA properties, receptor subunit expression is highly age-related (Fig.7). The adult subunit composition of the $GABA_A$ receptors in certain brain regions greatly differs from that in the embryo and neonate (e.g. cortex) and some receptor populations persist throughout development (e.g. hippocampus) while others proceed postnatally almost directly to an adult form (e.g. cerebellum). One of the most striking features of $GABA_A$ subunit expression during brain development is widespread mRNA expression of the α2 and α5 subunits with early peak and consequent decline. In contrast, α1 subunit expression increases. Expression of β1 subunit emulates that of α2 and α5 subunits, although at a much lower intensity. There were found no identical temporal and spatial patterns for two subunits expression. However, α1 and β2 expression often co-distribute while

α2, α3 and α5 expression often colocalize in time and region with β3 expression (Laurie et al., 1992).

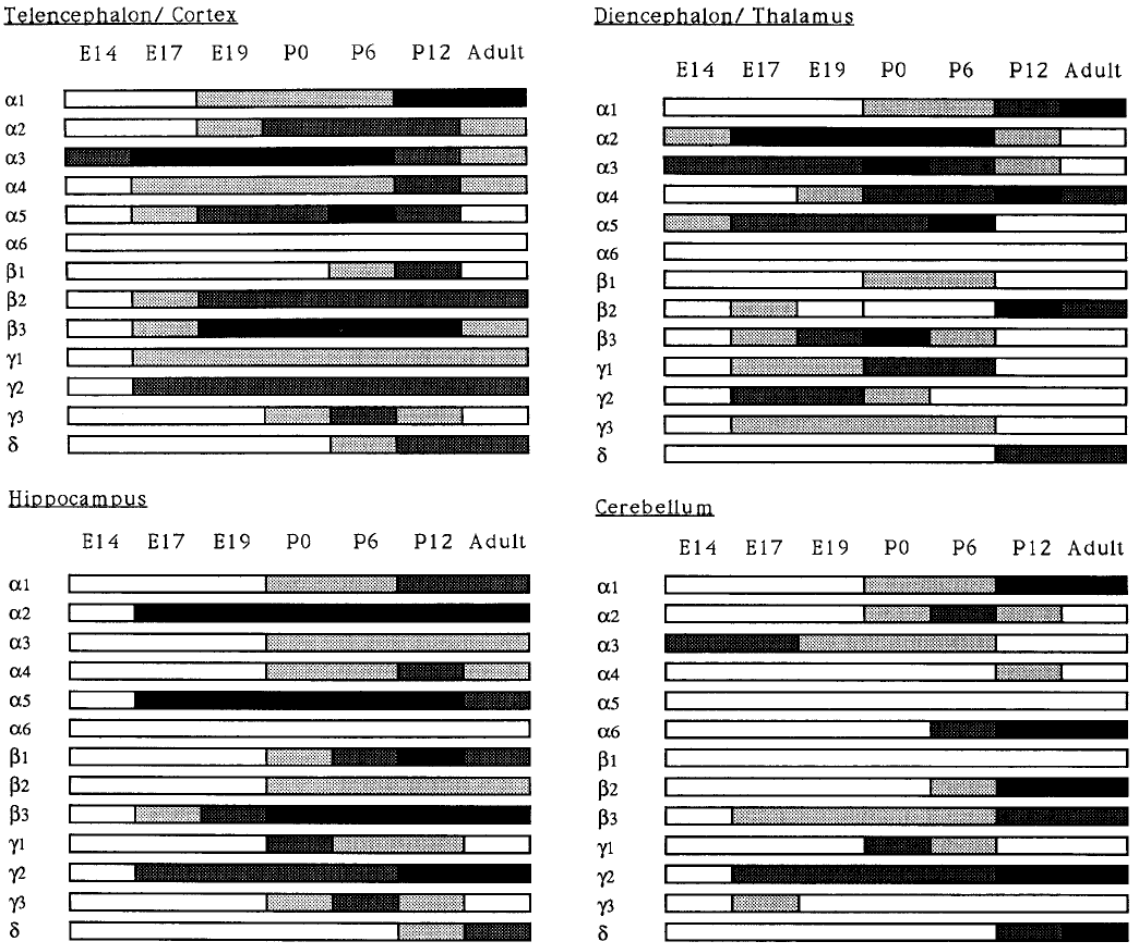

Fig.7 Expression of the $GABA_A$ receptor subunits mRNA in selected entire regions of the embryonic and postnatal rat brain. Black – strong signal, dark gray – moderate signal, light grey – weak signal, white – very weak or undetectable signal (taken from Laurie et al., 1992).

Developmental changes in the structure of $GABA_A$ receptors parallel with changes in function and pharmacology of these receptors. Hippocampal dentate granule cells (DGCs) and their $GABA_A$ receptors undergo marked changes in the pharmacological properties during the first 52 days of postnatal development. Functional studies demonstrate that maximal GABA-evoked current increases with age and that furosemide and zinc inhibit $GABA_A$ receptors currents in young rats but not in adult rats. In parallel, structure of $GABA_A$ receptors changes from type BZ3 in young rats to type BZ1 in adult rats ($GABA_A$ receptors has been traditionally divided according to pharmacological properties – BZ types differ in affinity for

certain BZs as zolpidem),and the fraction of cells expressing loreclezole sensitive $GABA_A$ receptors increased with age (Kapur and McDonald, 1999).

Changes in functional and pharmacological properties of $GABA_A$ receptors during development correlate with the changes in receptor subunit composition. The developmental changes in $GABA_A$ receptor subunits mRNA expression are associated with increase in α1 subunit expression, maximal GABA-evoked currents, and augmentation of GABA-currents by zoldipem as demonstrated in DGCs. In addition, sensitivity to zinc decreases with age (Brooks-Kayal et al., 2001).

2.2.5 Consequences of BZs treatment for development of $GABA_A$ receptor

Effects of BZs are mediated by $GABA_A$ receptors. Therefore, large number of studies has attempted to elucidate neurochemical changes associated with repeated BZ administration.

In adult rodents, chronic BZ treatment led to decreased maximal number of benzodiazepine binding site in the majority of experiments. In general, regional differences were found, with down-regulation of benzodiazepine binding site in the cortex, hippocampus and amygdala; least likely to be found in cerebellum or olfactory bulb. Importantly, results vary among experiments most likely due to the type of BZ, used dose and schedule of administration (Hutchinson et al., 1996). Studies concerning chronic BZs administration in adults have also reported changes in $GABA_A$ receptors subunit composition (Chen et al., 1999; Tietz et al., 1999).

The developing GABAergic system is highly plastic. Prenatal exposure to diazepam has been reported to change density of benzodiazepine binding site (Rothe and Bigl, 1989) and $GABA_A$ receptor structure (Roberts et al., 2001). Conversely, much less is known about long-term effects of early postnatal treatment with BZ. Sporadic study from Raol and colleagues (2005) observed changes in subunit composition in adult animals exposed to diazepam early during development . Treatment caused a significant decrease in the mRNA expression of α1 and α3 subunits and a significant increase in α4, α6, β3, δ and θ expression.

The specific properties of GABAergic system play the important role in the immature brain. Changes induced in this system by BZs exposure may lead to remodelation or alteration of structures of functional circuity and thus participate in functional changes in the developing brain.

2.3 Behavioral consequences of early benzodiazepine administration in experimental studies

It is known that children exposed to BZs pre- or postnatally experience undesirable effects of BZs such as poor gain weight and drowsiness. It was also suggested that exposure to BZs may have long-term adverse effects on neurobehavioral development, but hard data supporting this hypothesis are missing (Ebhard-Gran et al., 2005; Mizrahi and Clancy, 2000). In clinical studies, it is however difficult to separate effects of BZs from adverse effects of other drugs or alterations due to underlying pathologies. In addition, variability of studied subjects as well as social background may play important role in final outcome. Using experimental animals is a direct approach to address the question of whether the early BZs exposure without underlying pathology and additional treatment can lead to a reorganization of neuronal circuits, cognitive impairment, and alteration of social and/or emotional behavior. Most of our today knowledge about long-term effects of early BZs exposure comes from studies employing prenatal administration of BZs. Results of these studies suggest that prenatal BZs exposure could lead to behavioral deficits when tested as adults. Gai and Grimm (1982) found significant dose-dependent differences in a complex six-choice simultaneous discrimination learning task where diazepam-exposed rats made more errors and took more time to reach the goal. In open-field BZ rats showed longer latencies to enter field from starting box and lower rearing frequency (Gai and Grimm, 1982). Other studies dealing with prenatal BZs exposure showed various alterations including deficits in learning, active avoidance, conditioned place preference and exploratory behavior in various mazes, as well as disturbances of social behavior (Tucker, 1985; Alleva and Bignami, 1986).

Much less data are available to demonstrate long-term behavioral consequences after postnatal BZs exposure. Several studies suggested that BZs administered from P1 to P21 (till weaning) had significant effects on social behavior during adolescence. In the home-cage aggression test BZ animals displayed enhanced offensive/aggressive behaviors when they were resident in their home-cages, but increased submissive behaviors when the BZ-exposed rats were intruding into another rat's territory. All these changes in behavior were dose-dependent and highly affected by type of BZs tested (File, 1986 a, b, c). More recent experiment where pups received diazepam (from P2 to P21) was performed by Schroeder and collaborators (1997). When tested 2 months after the end of diazepam administration, animals displayed increased locomotor activity, reduced anxiety, but no learning or memory deficits. In all these studies, rats were exposed to BZs since they were born till weaning i.e. for

decisive part of development. Such experimental design makes very difficult to assess BZs-induced changes of early behavior separately from direct effects of these drugs.

2.4 Brain development and behavioral testing

2.4.1 Brain development in rodents

The correlation between the developmental stages of immature rodents and humans is indeed very complicated. At the time of birth, the rodent brain is very immature compared to that of human newborns. Rodents develop relatively quickly, but it still takes several weeks to reach fully mature stages of all biochemical, morphological and functional parameters. Using various molecular, biochemical, morphological, physiological, and behavioural methods, different studies have demonstrated the developmental time-course of individual variables. Therefore, even conclusions obtained in the same laboratory might vary in relation to the measured characteristic. For example, Adlard and colleagues (1973) claimed that a 5-day-old rat is an appropriate model for the human newborn in terms of brain maturation, having based their conclusion on the timing of the peak velocity of the accumulation of brain wet weight in both species. Based on the timing of the ”growth spurt” as a vulnerable-period, Dobbing (1970) compared human babies from the last few weeks of gestation through the first few months of life to rats 10 to 12 days old. In addition to biochemical parameters, the development of bioelectrical activity of the rat brain was studied and all the published data demonstrate that irregular EEG activity appears at the age of 5 to 6 days (Ellingson and Rose, 1970) and up to P10 EEG activity is interrupted by periods of electrical silence corresponding to the ”tracé alternant” described by Dreyfus-Brisac and collaborators (1964) in preterm newborns, but are never observed in full-term human newborns. In experimental epileptology, rats 10 to 12 days old are generally accepted to be in a stage of maturation corresponding with that of human newborns or early infancy. Physiological weaning in rats occurs around P28 and sexual maturation begins between P35 and P45 (Piacsek and Goodspeed, 1978).

2.4.2 Behavioral studies

Behavioral studies represent one of the possibilities how to investigate changes in the brain after early brain injury or exposure to a chemical agents and drugs. With proper

experimental design, they can be used to determine whether prenatal or postnatal drug exposure changes selected parameters of neurobehavioral development. Importantly, animal behavior studies can play essential role in assessment of possible risks created by exposures to doses or concentrations whose effects are below the threshold of detection of neuropathological methods.

Experimental design as well as selection of behavioral tests critically affects results obtained in these studies. There is enormous number of behavioral tests and their well described modifications available to map various behavioral parameters in adult rats. Behavioral studies are however less frequently performed in immature animals and specific tests developed for individual age groups of rodents are less known (Spear, 1990). Also, methodology of tests specifically developed for immature individuals is not unified and various laboratories are usually using slightly different modifications. However, it has to be emphasized that even small differences in methodology (selection of age groups, duration of drug exposure, experimental design, etc.) can substantially change the outcome of developmental studies. Thus in following paragraphs, background of behavioral tests used for present study will be summarized. To investigate effects of early clonazepam exposure on animal development and behavior, we exploited the native forms of behavior and stimuli that can be derived from the natural environment of rats.

Homing test

Homing test exploits the strong tendency of the rat pup to maintain body contact with the dam and siblings (i.e. the motivation is to reach the home), which requires adequate sensory and motor capabilities (Bignami, 1996). This test is well-suited to study spatial learning in immature rodents, since remembering home localization has a high adaptive value in weaning and preweaning animals (Schenk, 1989). It relays primarily on olfactory cues of animals. Preference for familiar nest odor is detected at P7 when rats become able to turn towards the familiar olfactory cue (Schlumph et al.,1989). The capability to return to home nest is highly relate to the maturation of motor skills (Altman and Sudarshan, 1975). Tendency to reach the home nest is preserved till weaning (Schlumph et al.,1989).

Social test

Rats establish complex societies and they are unquenstionably social animals (Meany and Stewart, 1979). In rats, periadolescence is reported as a critical period for development of social behavior which is characterized by an increased propensity to social interaction termed

as social play behavior. Rats start to play around P18, playing increases with age and peaks around P30-P36, and declines thereafter (Burghardt, 1999). The high rewarding value of social play behavior (Calcagnetti and Schechter, 1992) suggests its major importance for an animal's development. Social play behavior together with other social experiences in early life determines brain development and adult behavior (Van den Berg et al., 1999). The importance of early social experiences support the fact that animals may exhibit long-lasting behavioral changes if they are socially isolated during early life (Meany and Stewart, 1979) or exposed to various drugs during adolescence (Trezza and Vanderschuren, 2007). In addition to play related behavior, also behavior unrelated to play can be detected (for more details see Material and methods). These two forms of social behavior differ by their ontogenetic pattern because play behavior mainly occurs between weaning and puberty whereas other social behavior unrelated to play occur during the entire lifespan of rats (Vanderschuren et al., 1995).

Habituation test

Habituation, defined as a response decrement following repeated or continuos presentation of indifferent stimuli, represents a relatively simple form of long-lasting behavioral plasticity and one of the most elementary type of non-associative learning (Cerbone and Sadile, 1994). The open field test, which is a typical all-purpose observational test (Bignami, 1996) is usually used to assess locomotor activity and reaction to novelty. In this test, habituation is defined as a decrease of activity with time of exposure (short-time habituation) or with number of exposures (long-time habituation). It was shown that short-term habituation (i.e within session) can be for the first time observed at the end of the 3rd postnatal week, whereas long-term habituation (i.e between sessions) appears in normal rats at the end of the 4th week (Laviola et al., 1988).

Animal's performance in open field may markedly differ according to experimental conditions like intensity of illumination, background noise, and layout of landmarks outside the arena (e.g. the room furniture, the experimenter's location) (Bignami, 1996; Bouwknecht et al., 2007). Therefore, the same conditions must be preserved throughout the experiment.

In addition to assessment of locomotor activity and non-associative learning, open field test can be used to measure anxiety-related behavior (Bouwknecht et al., 2007).

Elevated plus-maze

This paradigm is based on a competition between internal drive to explore a novel environment (based on the potential for rewarding outcomes) versus the internal drive to avoid a novel environment (based on the potential for aversive outcomes), i.e. it is based on the apparent natural aversion of rodents to open and high spaces of open arms and the tendency of rodents to explore a novel environment. This test became very popular mostly because it is easy to perform and all measured parameters were well characterized. This model was validated as an animal model of anxiety in rats and mice by many pharmacological and behavioral studies (Lister, 1990). In addition to studies of anxiety and screening for anxiolytic drugs, Itoh and colleagues (1991) reported that the elevated plus-maze can be used to study learning and memory. They proved that transfer latency (i.e. time necessary to move from the open arms to the enclosed arm) is shortened if the animal has previously experienced entering the open arms. During the initial exposure an animal acquires phobic avoidance of the open arms and retains strong memory for this threat for a certain time. Thus, with slightly modified experimental protocol, this model can be used to study both anxiety-like behavior and cognitive abilities in rodents.

Morris water maze

Morris waster maze is extensively used to investigate specific aspects of spatial memory. This test is based on the premise that animal evolved an optimal strategy to explore the water maze and escape from the water with a minimum effort - i.e. animal swims the shortest possible distance to escape. A submerged (hidden) platform serving as the escape from the water offers no local cues to guide escape behavior and therefore extra-maze visual cues are necessary for orientation. The latency necessary to reach a hidden platform after previous exposure, is used as a criterion of spatial memory. Rats are natural swimmers and they can solve the water-maze task easily (Morris, 1984). Rats swim efficiently from P16, but they do not achieve a place navigation task in a water maze in the absence of any intramaze cue before P32-35 (Schenk, 1985). The spatial navigation is highly dependent on the hippocampal integrity. Thus, animals cannot solve this task before maturation of hippocampal structures (Pokorný and Yamamoto, 1981).

3. Aims of the Thesis

The goal of my thesis was to evaluate long-term effects of early clonazepam exposure on postnatal development and functional abilities of rats. Clonazepam was administered in five consecutive days starting at P7 to P11, i.e. during the critical developmental period of brain sprout. Based on available data we hypothesize that even short exposure to clinically relevant doses of clonazepam (Kubová and Mareš, 1989) during early postnatal development can cause long-term functional deficits. In order to test our hypothesis and to assess possible age-related alterations related to cognitive functions, motivation and emotionality animals will be tested repeatedly starting shortly after the end of administration till adulthood. Experiments were thus designed to study consequences of treatment not direct effects of clonazepam.

***Aims and scopes*:**

1. To assess effects of early clonazepam exposure on body growth and development.
2. To demonstrate whether early exposure to clonazepam leads to behavioral dysfunction related to cognitive functions, motivation and emotionality such as anxiety and fear
3. To characterize possible effects of early clonazepam exposure on social behavior

Present study is a part of project "*Long-term functional consequences of early benzodiazepine exposure in immature rats*" supported by grant No. 305/09/0846 of the Grant Agency of the Czech Republic

4. Material and methods

4.1 Animals

Experiment were performed using Wistar albino male rats (n=80). Rats derived from ten litters. On postnataly day 1 (PD1; birth counted as day 0) the pups were randomly fostered and each litter was adjusted to ten males. The animals were weaned at postnatal (P) 28. Rats were housed under a controlled environment (temperature 22±1 °C, humidity 50-60%, lights on 06.00-18.00 hours) with free access to water and food. The experiments were approved by the Animal Care and Use Committee of the Institute of Physiology of the Academy of Sciences of the Czech Republic. Animal care and experimental procedures were conducted in accordance with guidelines of the European Community Council directives 86/609/EEC.

4.2 Drug and treatment

Clonazepam (CZP; Hoffmann - Switzerland; got as a gift) was suspended in one drop of Tween 80 and 1ml of saline. Subsequently, this solution was diluted in such a way that various doses of clonazepam was administered in the same volume of 5 µl/g of body weight. Clonazepam was administred in three doses – 0.1 mg/kg, 0.5 mg/kg and 1.0 mg/kg intraperitoneally for five consecutive days starting at P7 till P11. Doses were selected according to our previous studies on anticonvulsive effects of clonazepam in developing animals (Kubová and Mareš, 1989). Control animals received a corresponding volume of saline.

4.3 Apparatuses

The homing test arena consisted of two transparent Plexiglas hexagonal cages (34 x 34 x 24 cm) connected by a small opening (diameter 4 cm). The home cage was divided in two equal parts by a transparent wall. The start cage was of the same dimension as the home cage. The pups were placed to the part of the home cage located farther from the start cage together with small amount of soiled bedding taken from their nest.

The open-field arena which was used for social and habituation tests consisted of a black plastic chamber (45 x 45 x 30 cm) with black floor.

The elevated plus-maze test was conducted in the maze made from black plastic and consisted of two open arms and two enclosed arms elevated 50 cm above the floor (each arm

was 10 cm wide and 50 cm long, extending from central platform 10 x 10 cm). Two open arms had low walls (1 cm), while the enclosed arms had walls 50 cm high.

The water-maze comprised of black circular pool (210 x 50 cm) filled with clear water (19-20^{0}C). A small transparent Plexiglas escape platform (10 cm in diameter) was placed in the center of an arbitrary defined quadrant of the pool (north-west). The platform was submerged 1.5 cm below the surface of the water and was maintained in a constant position throughout the experiment. Abundant extra–maze cues were available for spatial orientation.

4.4 Procedures

Tests were carried out in a special room with constant temperature (22±1 °C). Low-light conditions (35-45 lx) were used in all tests except from the water maze (100-110 lx) to prevent decreased behavioral activation and increased anxiety-related behavior. (Bouwknecht *et al.*, 2007) Background noise was produced by a fan in digital videorecorder which attenuated environmental disturbances. To reduce any lingering olfactory cues, every apparatus was cleaned after each rat. All tests were performed between 09.00 and 15.00 hours.

Behavioral tests started 24 h after the last CZP injection and continued up to the age of 4 months. The schedule of the whole study, i.e. timing of individual tests is summarized in Figure 8. Briefly, animals were exposed to the homing test four times, at the age of P12, P15, P18 and P23. Experiment continued with social test at P31 followed by one month long brake. Afterwards, animals performed habituation test for four consecutive days starting at P67. Animals were then tested in elevated plus maze for two times, at P74 and P75. Finally, animals were exposed to the water maze for five consecutive days beginning at P81.

Body weight was assessed daily during the administration of CZP, and later on at the beginning of each individual experiment.

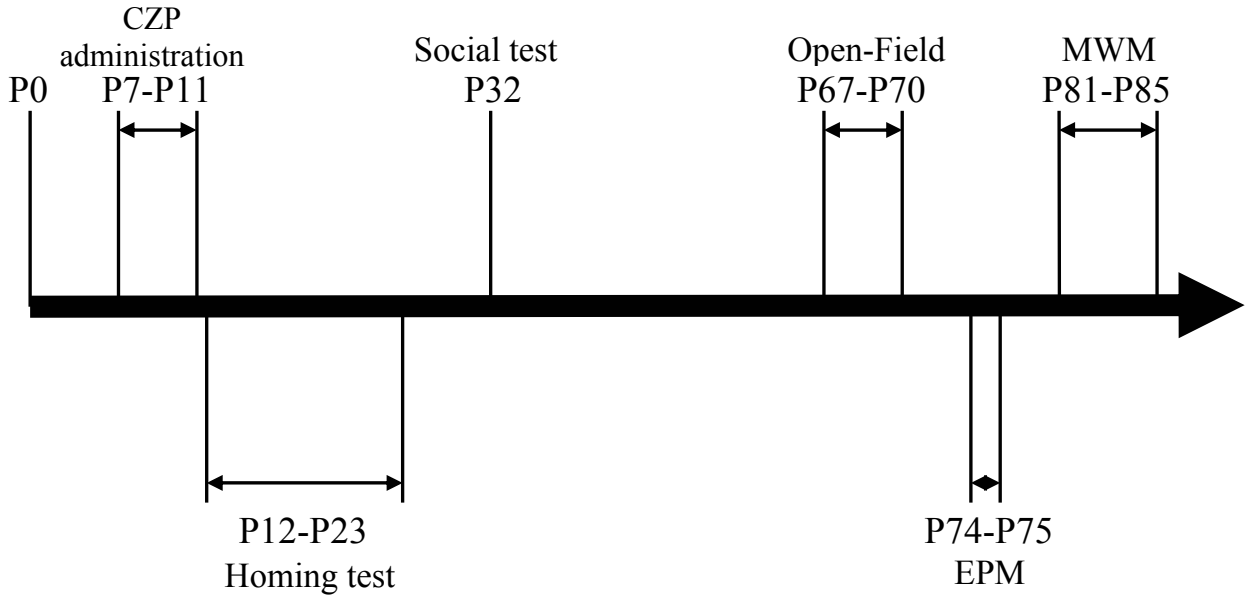

Fig.8 Time schedule of experiment

Homing test (Mikulecká and Mareš, 2009; Schenk, 1989)

Before the test pups were allowed to adapt to the new environment for 30min. Experimenat design was chosen according to previous study. (Mikulecká and Mareš, 2009) Briefly, twelve trials for P12 and 10 trials for P15, P18 and P23 rats, were used. Inter-trial intervals were 60s for P12 and P15 rats, 120s for P18 and 180s for P23 rats. The area of start cage was reduced to half for P12 rats because of decreased motor skills at this age. A correct homing response was considered if the pup was able to reach the home cage within 60s time interval. If the pup failed to enter the home cage, an experimenter gently aided him in direction to find the home cage.

The five consecutive correct responses were taken as a criterion for learned homing response.

Homing responses, i.e. average latency necessary to enter the home cage, and the ratio between correct responses and the total number of trials were analyzed. Moreover, differences in the same experimental group among single days were calculated.

Fig.9 Homing test, a pup is just entering a small hole between the start and the home cage

Social test (Corbett et al., 1991; Fraňková and Mikulecká, 1990; Vanderschuren et al., 1995)

The social test was performed after weaning (P28) at the age of 32 days. Each animal was placed to the open field arena for 5 minutes in two consecutive days before testing for adaptation. On the day of experiment, animals were sorted into pairs. Each pair consisted two unrelated animals from different litters and differences in body weight between animals never exceeded 10%. Then animals were put individually into plexiglas cages for social isolation for two hours. After isolation period, pair of animals was placed into the test cage for 10 min. Following two behavioral categories were analyzed:

1. Behavior related to play

- Pinning – this is defined as one of the animals lying with its dorsal surface on the floor of the test cage with the other animal standing over it
- Boxing/wrestling – a group of behaviors including boxing, wrestling, and pouncing
- Following/chasing – moving in the direction of or pursuing the test partner, who moves away

2. Behavior unrelated to play

- Social exploration – sniffing or licking any part of the body of the partner, including the anogenital area
- Contanct behavior – this type includes crawling over and under the test partner and social grooming (chewing and licking the fur of the partner, during which the animal that grooms mostly places its forepaws on the back or neck of the partner)

In addition, the number and the amount of time which two unfamiliar rats actively interact with each other in a familiar environment were measured. Therefore, the novelty of partner is the only stimulus which inhibits social behavior between the rats. Behavior was evaluated per pair of animals, which means that the behavior of an individual animal was not analyzed.

Fig.10 Two P32 rats in the open field arena during social test

Habituation test – Open field (Lister, 1990; Thiel et al., 1999)

Animals were tested on four consecutive days starting at P67. Thirty minutes before the onset of testing animals were transfer into the room where the test took place. Animals were placed into the center of the arena and their behavior was registered for 10 min. The following behavioral parameters were evaluated:

1. Locomotor activity – distance moved
2. Thigmotactic scanning – time spent locomoting along the walls of the arena (thigmotactic area was considered as an eight centimetres wide zone along the walls)
3. Centre time – time spent in the central area (central area was considered as zone surrounded by thigmotactic area)
4. Rearing – frequency and duration of vertical activity (irrespective of whether it occurred on or off the walls) – results not included in the thesis
5. Grooming behaviour – number and duration (considered as a complete sequence from paw licking and face wash through body wash and fur licking up to tail and genital licking and wash) – results not included in the thesis
6. The number of faecal boli was counted

Habituation of open field activity was investigated in two ways: within-session habituation (short-term habituation) was measured by comparing the 1st (0-5) vs 2nd (5-10) min interval of a given testing period. Between-session habituation (long-term habituation) was analyzed by comparing first day intervals vs. day four intervals.

Fig.11 The rat performing habituation test

Elevated plus maze (Itoh et al., 1991; Lister, 1990; Mikulecká et al., 2000)

Before the test, animals were transfere into the experimental room to acclimate for 30 min. The animals were tested on two consecutive days starting at P74. The first day is considered as the acquisition session, the second day is considered as the retention session. Each day they were exposed to the plus maze twice for 5 min each. At the beginning of testing, the animal was placed at the end of the open arm with head facing the central platform. The following behavioral parameters were registered :

1. Transfer latency – the time necessary to enter one of the enclosed arms (animals had to cross with all four legs an imaginary line separating the enclosed arm from the central platform) – expression of learning and memory
2. Total time spent on open arms per 5-min sessions expressed as percentage of total time of session – expression of anxiety
3. The number of enteries into closed arms, which expresses locomotor activity in the elevated plus maze

Fig.12 The elevated plus maze test. An animal stole from the enclosed arm and investigates surroundings

Morris water maze (Kubová et al., 2004; Morris, 1984)

The acquisition test was performed at the age of P81-P85. The rats were brought to the experimental room at least 30 min before experiment in order to acclimate. Each rat received one training session (8 swims) per day for 5 consecutive days. A trial began by placing the animal into 1 of 4 randomly selected sectors (north, west, south, or east; each starting point was used no more than 2 times per session) with the head facing towards the wall. The rat was allowed to swim until it found the escape platform or had swum unsuccessfully for 60 s. In the latter case, the rat was guided to the platform by hand and its latency in that exposure was recorded as 60 s. Animals were let to rest on the platform for 30-s. After each daily session the rats were dried with a towel and kept in a warmed cage. When their fur was dry they were moved back into home cages.

To evaluate the long-term memory animals were re-tested ten and forty days after the end of acquisition test. Only one training session was used for this retention test.

The following parametres were analyzed:

1. Escape latency – time per session which is needed for finding and climbing onto the submerged platform
2. Correct/total trials – the ratio of succesful trials to the total number of given trials
3. The long-term memory was assessed by comparing differences in escape latency of the last session of the acquisition test with latency from retention sessions – results not included in the thesis

Fig.13 The Morris water maze test. After reaching submerged platform, the rat is resting and investigating surrounding area. The extra-maze visual cues (black rectangle and triangle) are mounted on the walls. The digital camera is mounted above the pool and serves for the recording of sessions. It may be exploited as an visual cue as well.

4.5 Evaluation

All the tests were recorded using a digital camera (Panasonic WV-BP 330/GE, China) and a digital recorder (Samsung SHR-2042, Japan). Subsequently, recordings were evaluated offline by means of software Ethovision XT and Observer XT (Noldus Information Techology, Wageningen, The Netherlands). Ethovision XT is the software platform for automated tracking and analysis of animal movement and activity. Observer XT is the professional software for the collection and analysis of observational data.

4.6 Statistical analysis

Parameters were analyzed using SigmaStat® (SPSS Inc., Chicago, IL) software. All parameters were calculated with non-parametric tests. The data are expressed as mean ± SEM. A p-value of less than 0.05 was considered to be significant.

Body weight was expressed as a percentage of the starting value (weight at P7 was taken as 100%). The relative body weight ($W_{rel}=W_{P(n)}/W_{P7}$ x 100) were calculated to minimize effect of variability. To compare weights among groups the Kruskal-Wallis analysis was used.

Kruskal-Wallis analysis with post-hoc Dunn's method was used in homing test, social test, habituation test, elevated plus maze test and Morris water maze for comparison of groups.

Friedman repeated measures analysis of variance on ranks followed by Dunn's method was calculated to compare differences within group in homing test and Morris water maze.

Mann-Whitney test was used in habituation test for comparison of two days in a given period and within group.

5. Results

5.1 Effect of clonazepam on morbidity and body weight gain

Table 1 summarizes the numbers of animals used for present study. One pup assigned to CZP 0.1 mg/kg group died before the start of clonazepam administration. Mortality during CZP administration was very low (3 of 79 animals) compared to previously published studies (File, 1986 a). This can be explained by relatively low doses and short period of administration and also due to experienced dams used in this study. Therefore pups treated with clonazepam survived in spite of the lower weight gain.

Drug group	No. of animals in groups	No. of deaths after CZP treatment	No. of tested animals
Control	20	0	20
Clonazepam			
0.1 mg/kg	19	2	17
0.5 mg/kg	20	1	19
1.0 mg/kg	20	0	20

Table 1 Overview of animals assigned to experimental groups

Before the drug administration at P7, animal weight was assessed and the average weight was 18 ± 0.15 g in every group and there were no differences before administration onset. In control group, body weight gain regularly increased between two consecutive days since the onset of experiment (Fig.14, 15). In CZP-exposed animals, relative body weight was significantly lower during drug administration in all dose groups ($P<0.001$). After the end of CZP administration, significant differences in relative body weight occurred in CZP 0.5 mg/kg group till P18; in animals receiving CZP in a dose of 1 mg/kg body weight was lower till the end of study.

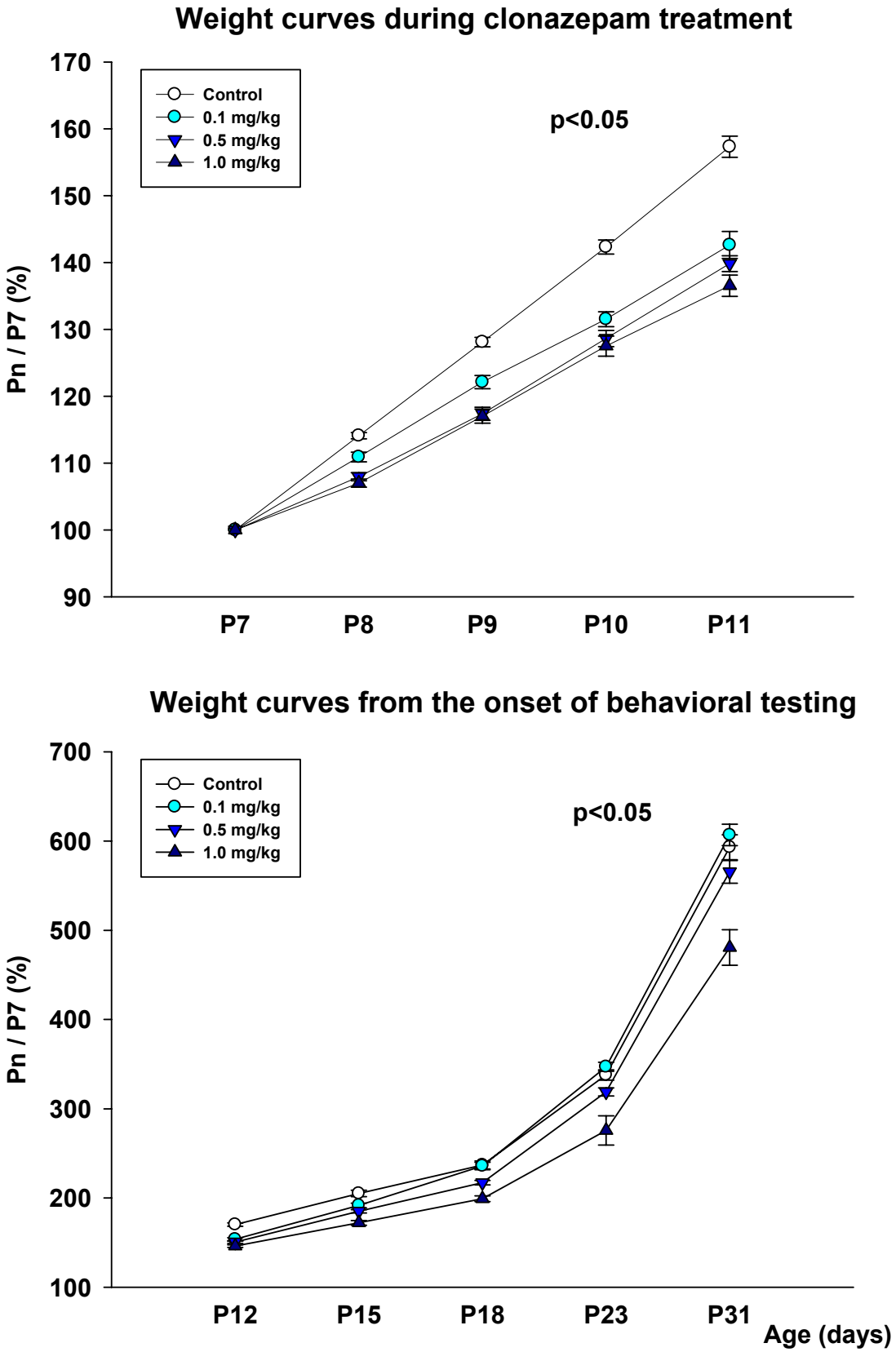

Fig.14 Top graph - Relative body weight of rats during clonazepam treatment (P7-P11); Bottom graph - Relative body weight of rats from the onset of testing, i.e P12. Y-axis: relative body weight (body weight on the day of first injection-P7 was taken as 100%), X-axis: postnatal days in which body weight was measured

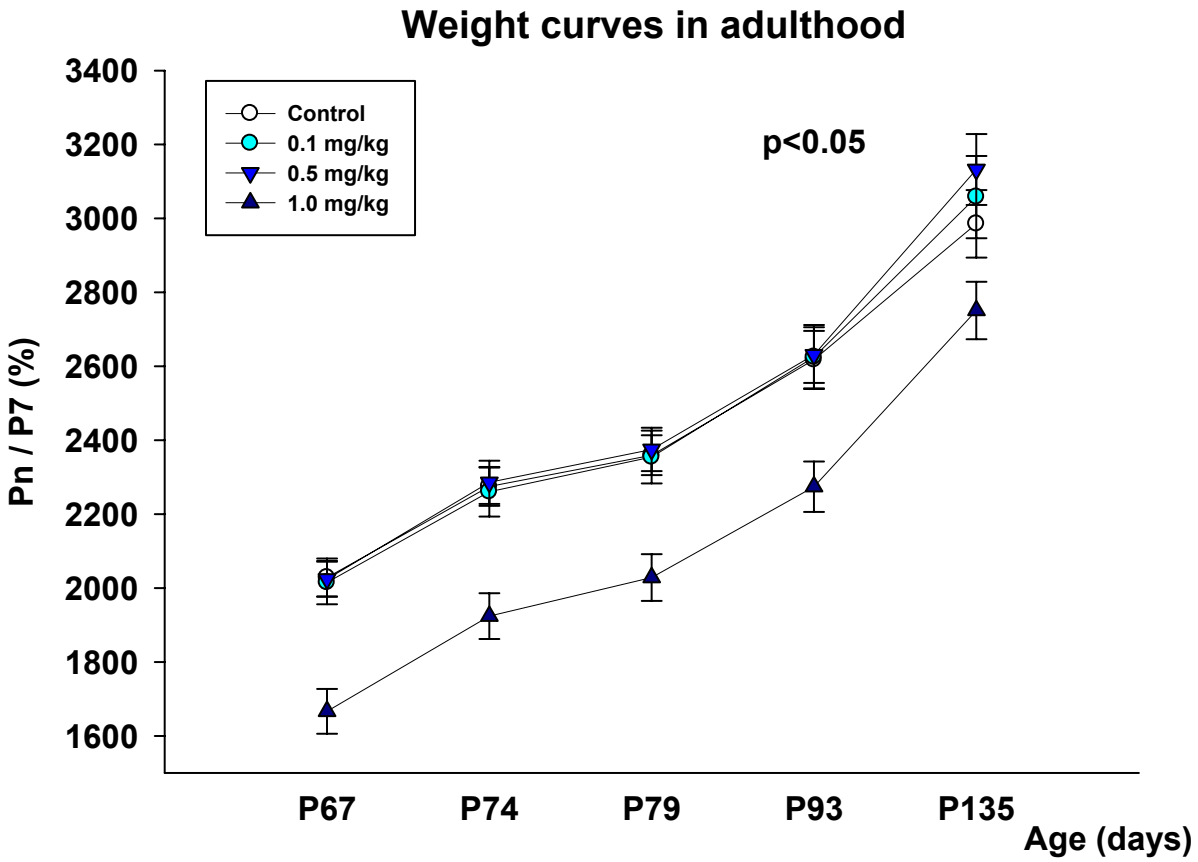

Fig.15 Relative body weight of rats in adulthood. Y-axis: relative body weight (body weight on the day of first injection-P7 was taken as 100%), X-axis: postnatal days in which body weight was measured

5.2 Effect of clonazepam in the homing test

In controls, homing response gradually shortened with number of tests (Fig.16). Along with decrease of latencies to home cage, percentage of correct responses increased (Fig.17). These changes were significant since P18 (Chi2=35.580, P<0.001 – homing response; Chi2=35.016, P<0.001 – correct responses ratio). Similarly, the improvement expressed as shortening of homing response and increase of correct responses was observed in all CZP-exposed groups. CZP 0.1 mg/kg group showed shorter latencies and higher percentage of correct responses since P18 (Chi2=18.459, P<0.001 and Chi2=26.064, P<0.001, respectively). CZP 0.5 and 1.0 mg/kg groups had significantly improved results since P15 (CZP 0.5 mg/kg : Chi2=34.111, P<0.001 – homing response; Chi2=36.865, P<0.001 – correct responses ratio; CZP 1.0 mg/kg : Chi2 41.984, P<0.001 – homing response; Chi2=39.686, P0<0.001 – correct responses ratio)

In the first test performed 24 hours after the last drug administration, homing response and percentage of correct responses was shorter in controls compared to animals receiving CZP in doses of 0.1 and 1.0 mg/kg (H=17.417, P<0.001 – homing response; H=19.585, P<0.001 – correct responses ratio). While in controls mean homing response was 40.5 ± 1.9 seconds, in CZP 0.1 mg/kg group was response 49.6 ± 2.2 seconds, and in CZP 1.0 mg/kg group 52.6 ± 1.3 seconds. Correct response ratio was also low – 38.2 ± 6.4 % in CZP 0.1 mg/kg and 31.3 ± 5.1 % in CZP 1.0 mg/kg, whereas in controls 66.3 ± 4.0 %. There was no difference in any of evaluated parameters in following tests. However, there was clear tendency to longer homing response as well as lower percentage of correct in both CZP 0.1 and 1.0 mg/kg groups at P23. 10 out of 20 rats from CZP 1.0 mg/kg group had the homing response longer than 30 seconds (P=0.041) and 5 out of 20 rats had correct responses ratio equal/lower than 60% compare to control (P=0.047).

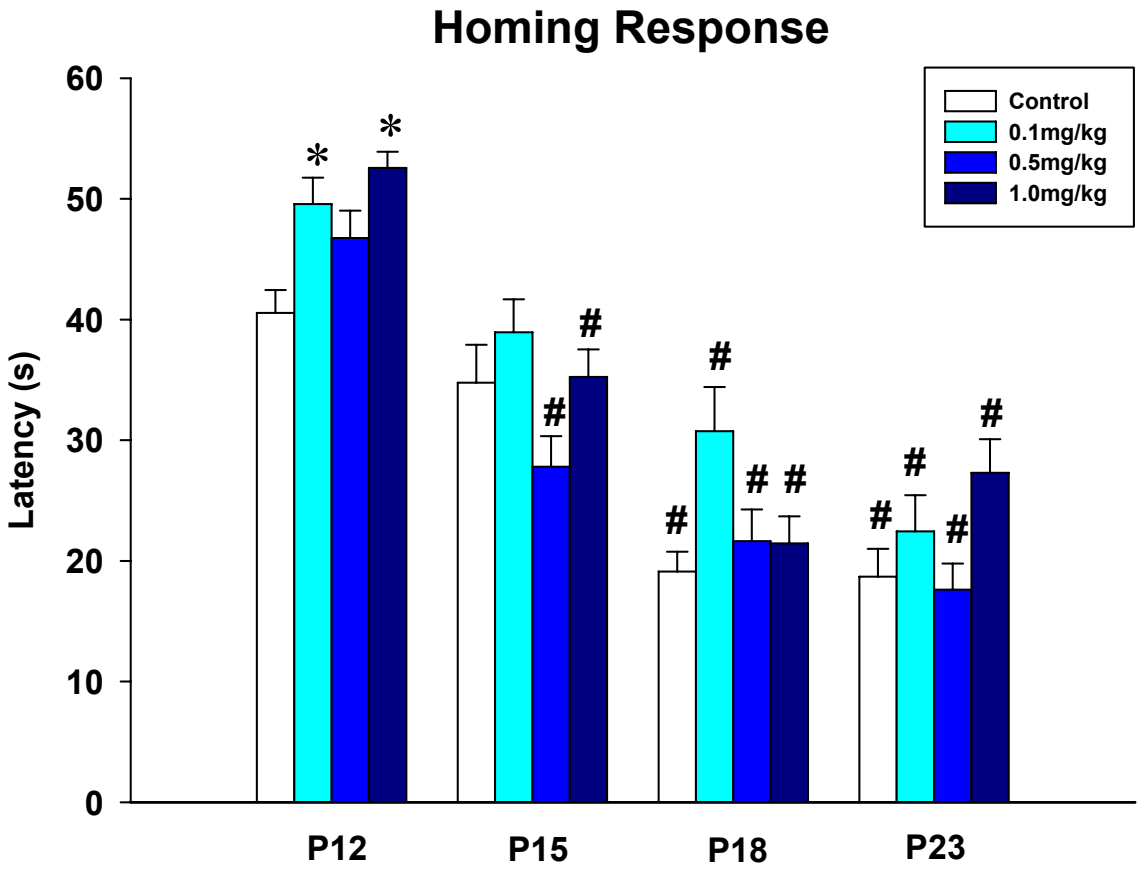

Fig.16 Performances in the homing test – homing response. X-axis: postnatal days in which homing test was performed. Asterisks mark a significant difference in comparison with corresponding control groups, double crosses mark a significant differences in comparison with P12 animals.

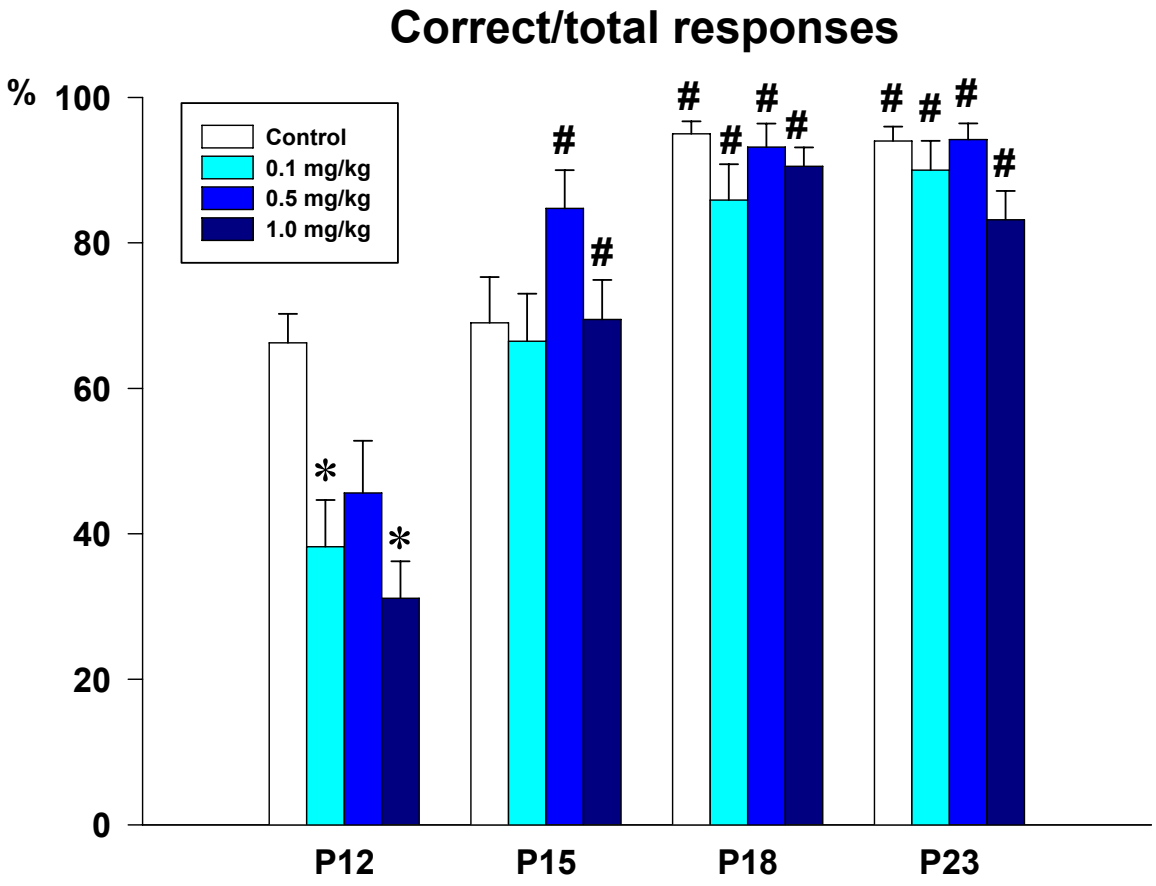

Fig.17 Performances in the homing test – ratio of correct to total responses. X-axis: postnatal days in which homing test was performed. Abscissa: percentage of correct responses. Asterisks mark a significant difference in comparison with corresponding control group, double crosses mark a significant differences in comparison with P12 animals.

5.3 Effects of clonazepam in the social test

Early CZP exposure affected behavior related to play (Fig.18). There was no difference in time spent with behavior related to play between controls and CZP 0.1 mg/kg group (13.6% vs.12.8%). Animals receiving CZP in doses of 0.5 and 1.0 mg/kg spent significantly shorter time playing compared to controls (2.5% and 2.8%, respectively; H=12.136, P=0.007). There was also tendency to decrease time spent with behavior unrelated to play (all CZP-exposed animals spent approximately 30% less time compare to controls). This differences however did not reached level of significance.

Typically, behavior related to play was characterized by longer duration and less frequent contacts between tested animals, whereas behavior unrelated to play had short duration and more frequent contacts. The number of contacts in both measured types of behavior was highest in controls. In both CZP 0.5 and 1.0 mg/kg groups frequency of contacts during play was lower compared to controls (H=14.795, P=0.002). CZP 0.1 mg/kg group did not differ from controls in this parameter. In behavior unrelated to play, number of contacts was significantly lower only in animals receiving the highes dose of CZP (1.0 mg/kg; (H=10.032, P=0.018). See Fig.19

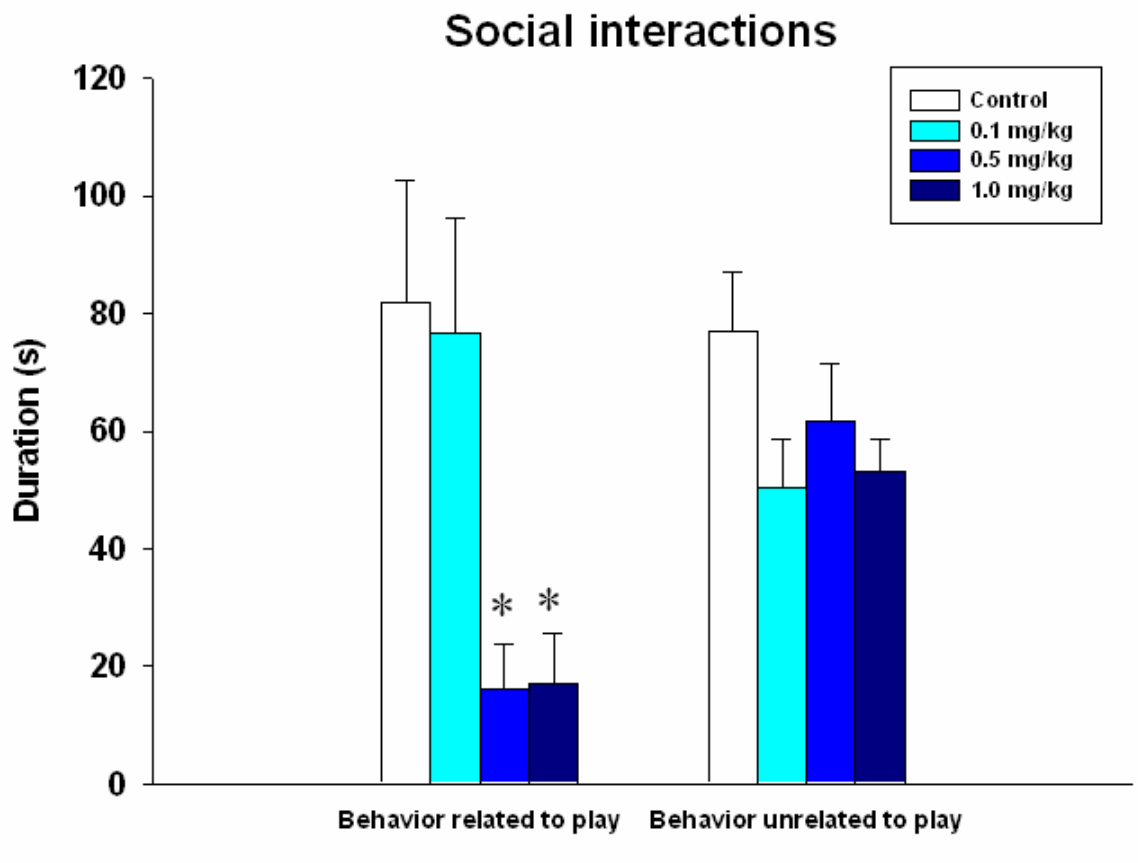

Fig.18 Performances in the social test. Graph shows duration of two observed types of behavior. Asterisks mark a significant difference in comparison with control group

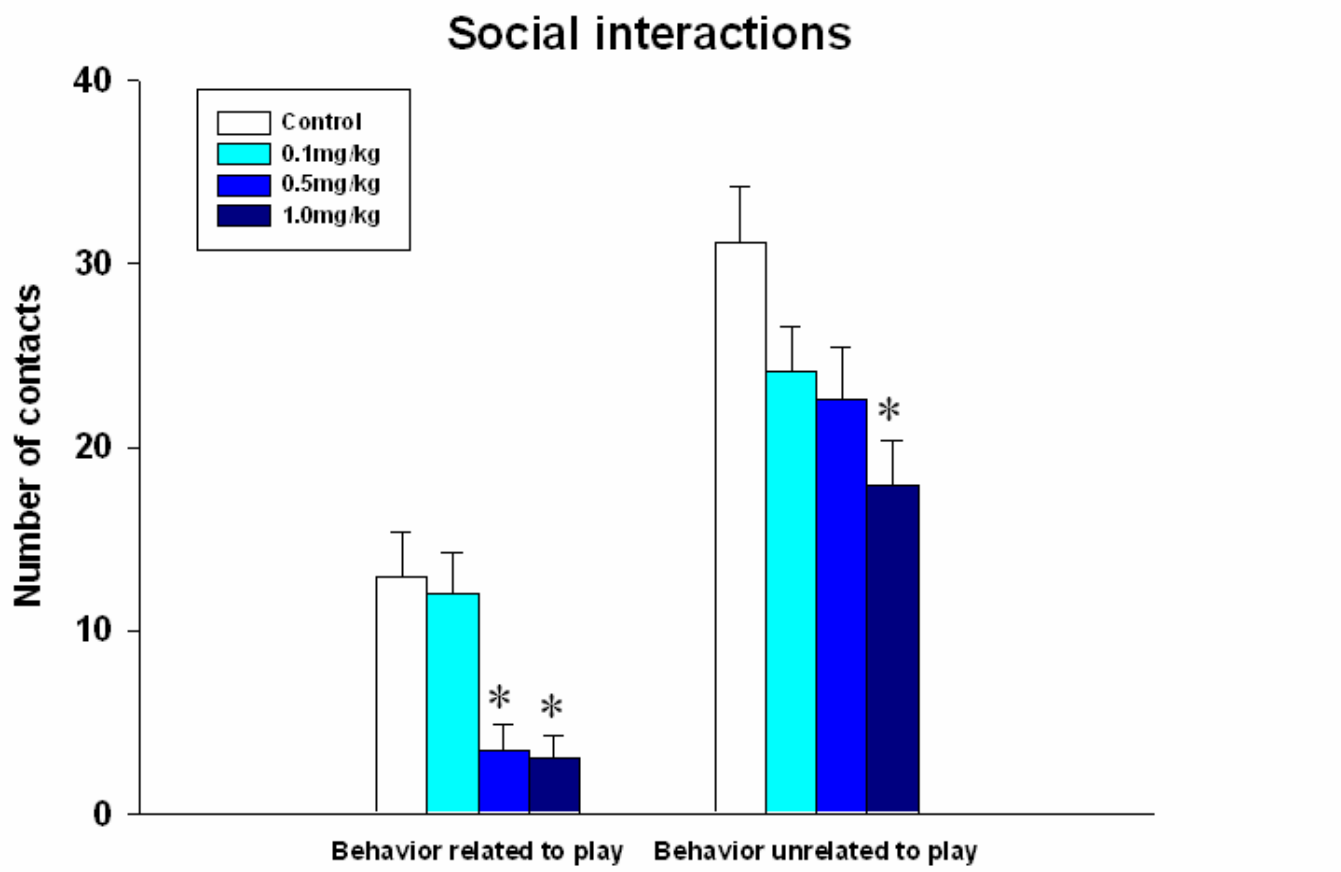

Fig.19 Performances in the social test. Graph depicts the number of animal contacts during social test. Asterisks mark a significant difference in comparison with control group

5.4 Effects of clonazepam in the habituation test

During the 1st test (i.e. during the 1st exposure to the open field arena) there was no difference in locomotor activity between controls and CZP-exposed animals in either 5-min interval evaluated. (Fig.20). There was the tendency to decrease the travel distance between the 1st and 2nd 5-min interval, but the differences were not significant. Also, during the 4th test, there was no difference in travel distance between controls and CZP-exposed animals in any of 5-min intervals. Travel distance decreased in the 2nd 5-min interval compared to the 1st one in both control and CZP exposed groups. (Control – $U=38.0$, $P<0.001$; CZP 0.1 mg/kg – $U=43.0$, $P=0.012$; CZP 0.5 mg/kg – $U=60.0$, $P=0.001$; CZP 1.0 mg/kg – $U=84.0$, $P=0.002$).

Comparison of travel distance during the 1st 5-min interval between the 1st and 4th tests showed significant increase in CZP 0.5 mg/kg group ($U=227.0$, $P=0.041$). Increase seen in CZP 0.1 mg/kg group was not significant. Decrease of travel distance between the 1st and 2nd 5-min intervals occurred only in controls ($U=225.0$, $P=0.040$), but not in CZP-exposed

animals. Long-term habituation was impaired, whereas short-term habituation was not affected.

In the 1st test, controls spent 97% of time the during the 1st 5-min interval by thigmotactic scanning. Data analysis showed that CZP 0.1 mg/kg group spent thigmotactic scanning significantly shorter time (90%) (H=13.223, P=0.004). In the whole 4th test, there were no differences between controls and CZP. CZP 0.5 mg/kg group spent less time along the walls when compared both intervals between the 1st and 4th tests (1st 5-min – U=62.0, P=0.002; 2nd 5-min – U=98.0, P=0.045). See Fig.21

Changes in time spent in the central zone of the open field arena parallel those from thigmotactic scanning analysis (Fig.21).

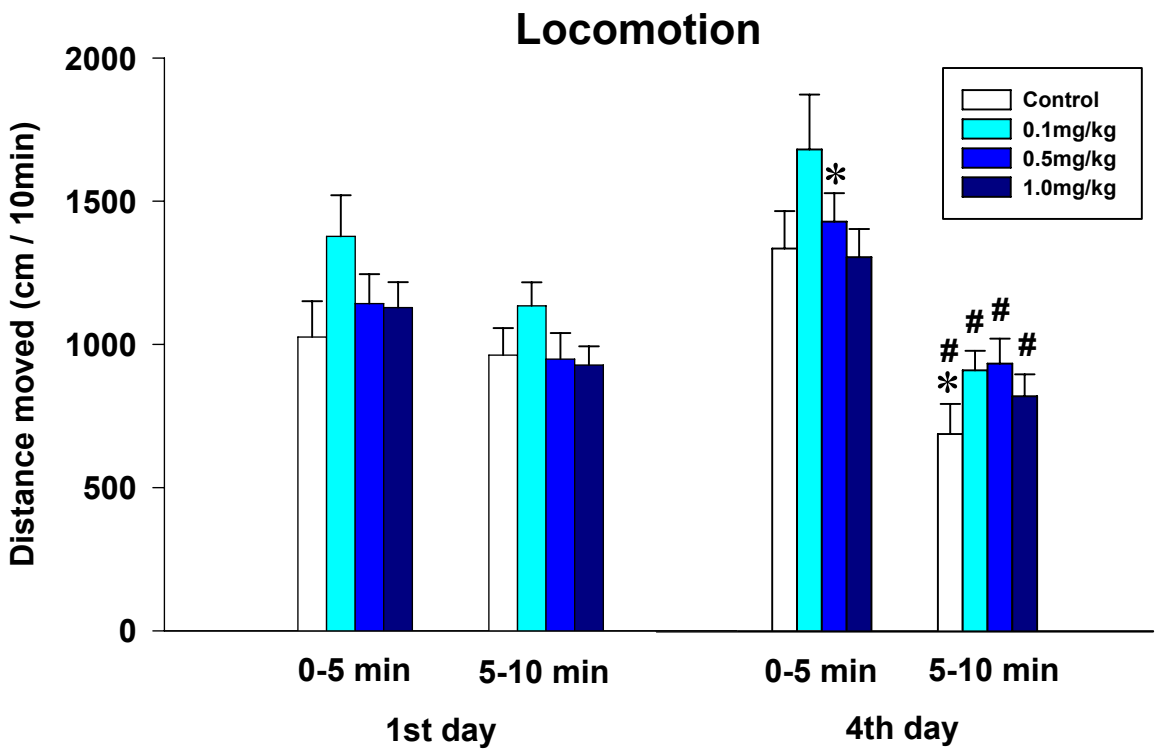

Fig.20 Performances in the habituation test - travelled distance during first and fourth day. Asterisks mark significant differences in given periods between day one and day four. Double crosses mark significant differences between two periods on day four.

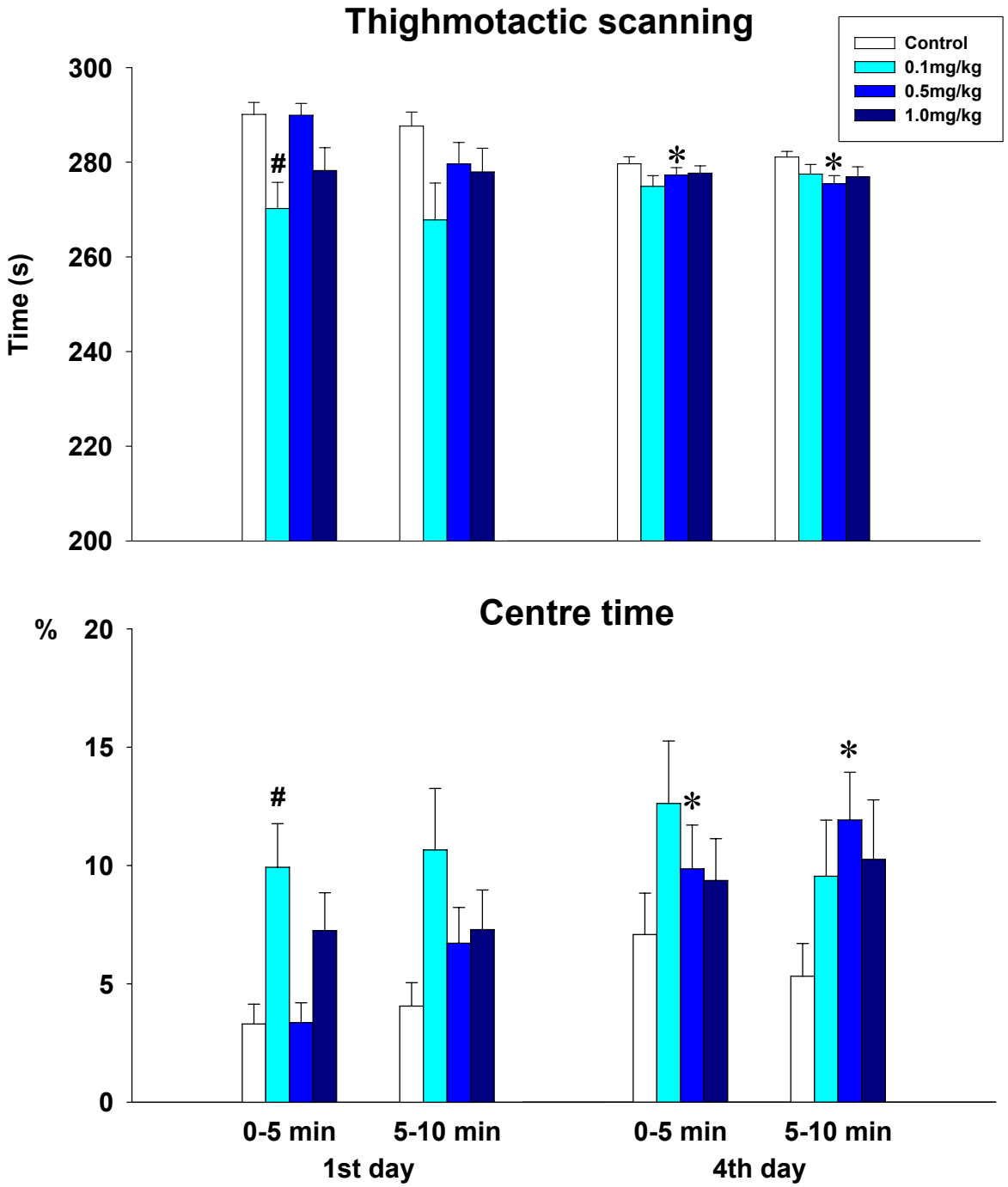

Fig.21 Performances in the habituation test – top graph: time spent with thigmotactic scanning; bottom graph: time spent in center. Abscissa for the bottom graph: percentage of time spent in the center area of open-field arena. Asterisks mark significant differences in given periods between day one and day four. Double crosses mark significant differences in comparison with control group.

5.5 Effects of clonazepam in the elevated plus maze test

The transfer latency between open and closed arms (Fig. 22) did not differ between controls and CZP groups in any testing session. Transfer latency decreased between the 1st and 2nd session in both controls (U=43.0, P<0.001) and all CZP-exposed animals (CZP 0.1 mg/kg – U=58.0, P=0.003; CZP 0.5 mg/kg – U=58.0, P<0.001; CZP 1.0 mg/kg – U=62.5, P<0.001)

Early CZP exposure did not affect time spent on open arms in any session (Fig X) and significant decrease of time was achieved in both controls (U=80.0, P=0.002) and CZP-exposed animals (CZP 0.1 mg/kg – U=80.0, P=0.027; CZP 0.5 mg/kg – U=85.0, P=0.006) except for CZP 1.0 mg/kg group in which differences did not reached level of significance (Fig.23).

There was no difference in the number of entries into closed arms between controls and CZP-exposed animals (Fig.23)

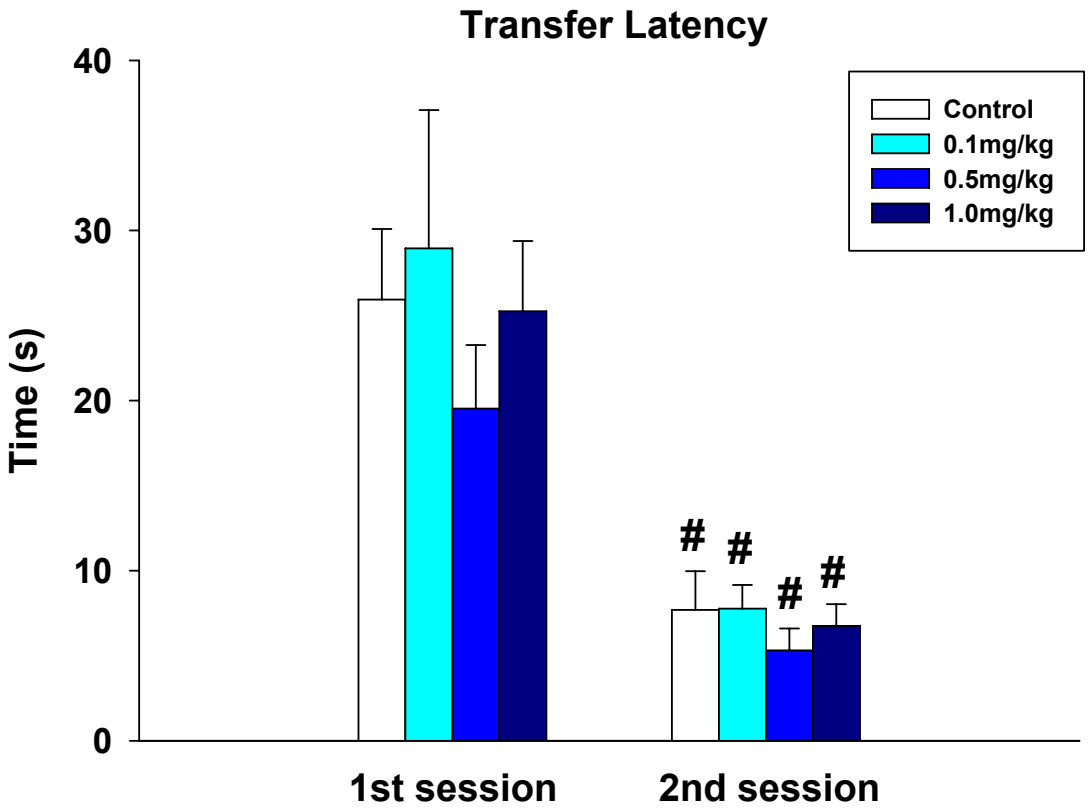

Fig.22 Performances in the EPM test – transfer latency to the enclosed arm of the EPM. Double crosses mark significant differences between 1st and 2nd session in a given group.

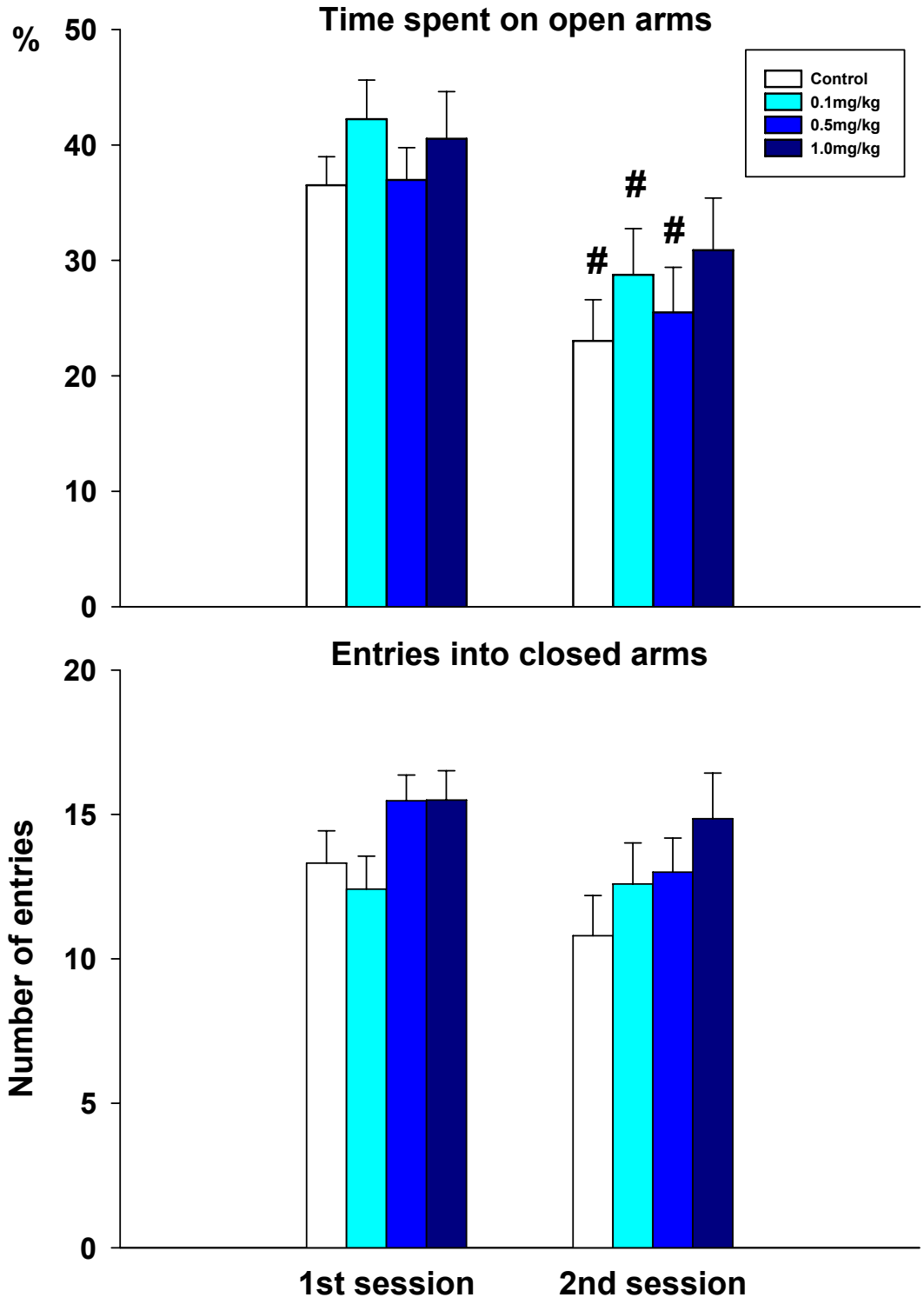

Fig.23 Performances in the EPM test. Top graph: time spent on open arms; Bottom graph: number of entries into closed arms. Abscissa for the top graph: percentage of time in a session spent on open arm. Double crosses mark significant differences between 1st and 2nd session in a given group.

5.6 Effects of clonazepam in Morris water maze test

All animals were able to solve this task and escape latency, i.e. time necessary to reach hidden platform, gradually decreased with number of session (Controls: Chi2=41.293, P<0.001; . CZP 0.1 mg/kg – Chi2=32.412, P<0.001; CZP 0.5 mg/kg – Chi2=64.253, P<0.001; CZP 1.0 mg/kg – Chi2 48.648, P<0.001). The overall analysis did not reveal any differences between controls and CZP groups in any sessions (Fig.24).

The ability to find hidden platform expressed as the correct/total trial ratio was not affected by early CZP exposure, however, the overall analysis approached the level significance in all sessions and reached significance in the 4th session (H=8.380; P=0.039), but post-hoc comparison did not reveal any differences. (Fig.25)

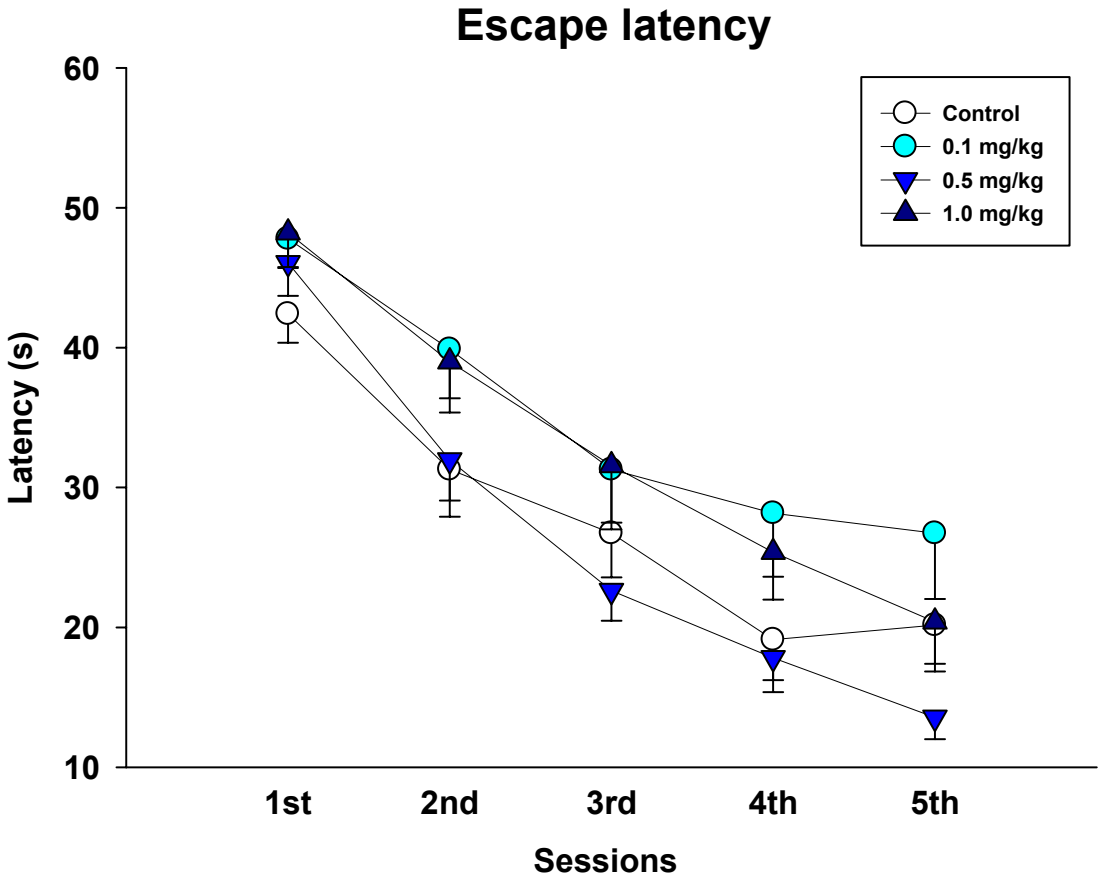

Fig.24 Performances in the Morris water maze test - escape latency.

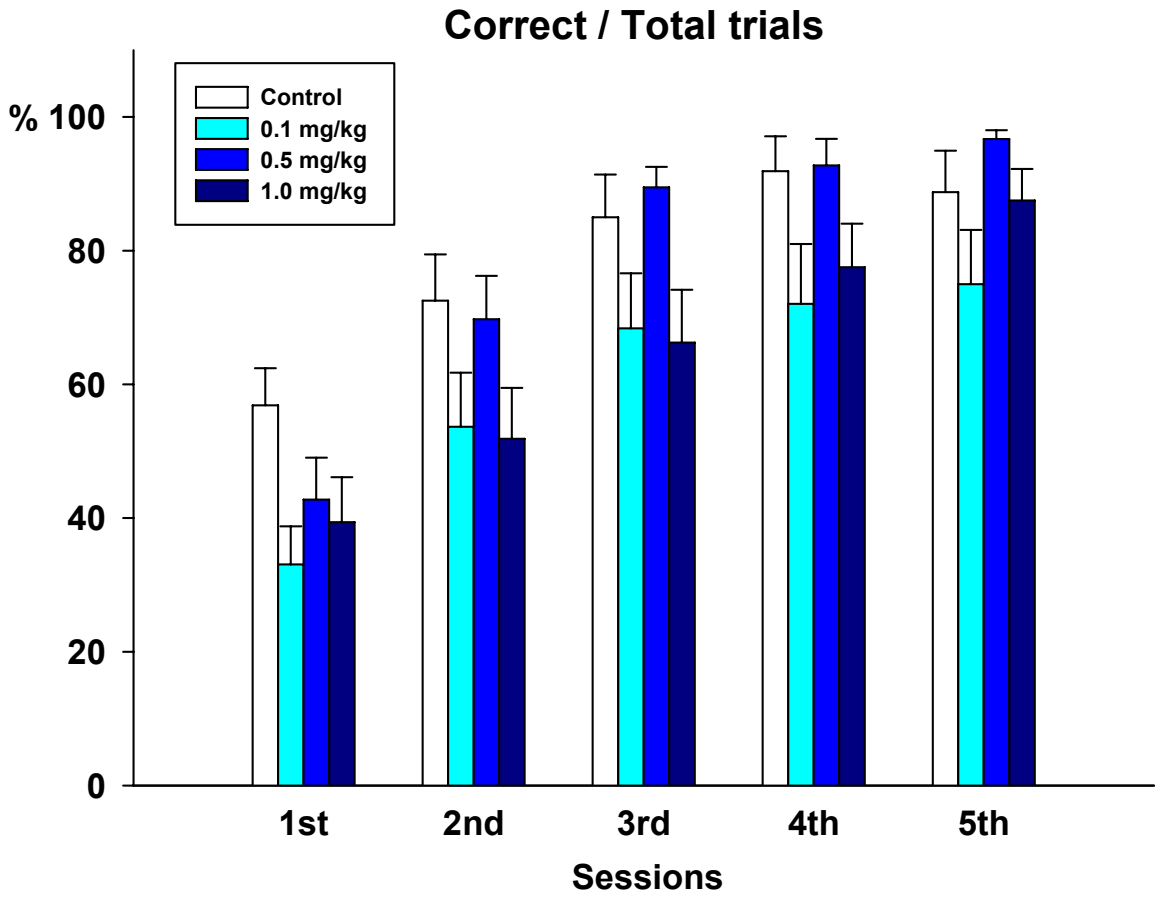

Fig.25 Performances in the Morris water maze test – ratio of correct to total trials Abscissa: percentage of correct trials.

6. Discussion

Present data show that even short exposure to clinically relevant doses of clonazepam during the critical developmental period can affect anxiety-like and emotional behavior as well as social behavior of rats later in the life. When administered between P7-P11, CZP led to mild and transient changes in the homing response, age-specific test of memory and learning abilities, changes of anxiety-related behavior in both open field and elevated plus maze and subtle changes of behavior in the Morris water maze test. Early CZP exposure affected most markedly social behavior. In social test, specifically selected for periadolescent animals, early CZP exposure suppressed behavior related to play and markedly influenced behavior unrelated to play.

Since their introduction, BZs have become one of the most frequently prescribed psychotropic drugs for their hypnotic, sedative, anxiolytic, muscle-relaxant and anticonvulsant properties and large therapeutic index. As highly effective tranquilizers and sedatives, these drugs are given to pregnant women (Heinonen, 1977) and they are also used in newborns (even premature), infants and children particularly for their anticonvulsant effects (for rev. Browne and Penry, 1973). Thus, possible unfavorable effects of early exposure to these drugs have been of great concern. For long time, BZs were considered to be relatively safe even for very young individuals, but after longer experience with these drugs the risk of behavioral teratogenicity became more obvious. Today, BZs are less frequently used in pediatric psychiatry, but they still belong to drugs of choice in treatment of childhood epilepsies or age-related epileptic syndromes. As antiepileptic drugs, BZs are usually used only for limited period of time, thus the immature brain is exposed to their effects only for certain developmental period (Dulac, 1994).

Long-term drug-induced impairment is difficult to study in clinics because of heterogenicity of studied subjects, underlying pathologies, various genetic background etc. Using experimental models in healthy immature rats is a direct approach to address the question of whether early drug exposure without underlying pathology (*e.g.,* brain damage, epilepsy) can lead to a reorganization of neuronal circuits, behavioral alterations, and cognitive decline. It has to be emphasized that so far published studies usually used very long exposure period, covering whole period since birth till weaning, or prenatal and postnatal period of development (Gai and Grimm 1982; Frieder et al., 1984; Schroeder et al., 1997; Tucker, 1985). In addition, results of these studies are also highly dependent on used

benzodiazepine type and dose, and selected behavioral tests/ measured parameters and intervals after the end of exposure (Tucker, 1985).

For present study, behavioral tests were chosen based on previously experiences obtained in animals with early brain injury (status epilepticus, ischemia) or with rat pups subjected to antiepileptic drugs (Kubová et al., 2004; Mátéffyová et al., 2006; Mikulecká and Mareš, 2009; Mikulecká et al., 2000). Used doses of clonazepam are clinically relevant, i.e they were found to prevent the development of acquired seizures in rat pups (Kubová and Mareš, 1989). At the same time, the highest dose of CZP used for present study, 1 mg/kg, significantly affects senzorimotor functions and ultrasonic vocalization of P12 rats after single administration (Mikulecká et al., in preparation). Whereas anticonvulsive effects of CZP administered in a dose of 1 mg/kg are still detectable after 24 h (Kubová and Mareš, 1989; Kubová, unpublished), sedative side effects last for shorter time (Mikulecká, unpublished).

In present study, repeated exposure to CZP in all tested doses led to decrease of body weight gain during administration. Animals however were gaining some weight even during this period. It means that even during administration of the highest dose tested animals were not starving or dehydrated. It has to be mentioned that BZs noticeably attenuate the ultrasonic vocalization in the rat pup which is considered as the distress call necessary for maternal retrieval (Gardner, 1985; Naito et al., 1998). Thus, CZP-exposed rat pups probably emited less ultrasonic signals which might result in poorer maternal care and lower weight gain. BZs including CZP have sedative effects in adult animals (File, 1981) and even though data for immature rats are very limited, single administration of CZP in tested doses is sedative also in developing rats (Mikulecká, unpublished). Thus, temporary growth retardation can be rather due to decreased ability to suck than due to lack of maternal care. Our experience speaks in favor of this explanation because random observations did not confirm maternal rejection or obvious lack of maternal attention during CZP administration. In addition, retardation in the growth was detected only for limited time period. Previous studies on the influence of malnutrition on growth of body and brain demonstrated that decrease in food supply for lactating dams results in retardation of general growth of pups. By the age of P21, these undernourished pups had reached less than 75% of the normal growth. Importantly, there was no difference in the wet weight of the brain between normal and undernourished rats during pre-weaning period and it cannot be presumed that it had a long-term effect (Dobbing et al, 1971). Therefore, we consider it unlikely that behavioral changes detected in CZP-exposed rats are related to the transient growth retardation.

Our study revealed differences between controls and CZP-exposed animals in the homing tests, which is designed to study spatial learning and memory in immature rats (Schenk, 1989). Rats tested at P12 exhibited clear functional impairment in this test because they needed longer time to reach home cage and at the same time their success rate was lower than in controls. These alterations are probably due to the fact that CZP is not yet fully eliminated and some effects can still be detected even 24 h after administration as discussed before (Kubová and Mareš, 1989). In adult rats, acute BZs administration leads to reduction in spontaneous activity, but chronic treatment leads to rapid development of tolerance to sedative effect (File, 1985). Data on adverse effects of BZs in immature animals are sparse, but our previous studies suggest sedative effects also in rat pups (Kubová et al., 1999). Thus, failure in homing test seen 24 h after the last CZP administration can be interpreted as an acute adverse effect of CZP rather that cognitive impairment or functional developmental delay due to early CZP exposure. When tested at P23, CZP 1 mg/kg animals tended to prolong homing latencies and to decrease percentage of correct responses. Even though this tendency was not significant, number of animals exhibiting alteration in this test (i.e. homing latency longer than 30 s and correct response ratio equal or higher than 60%) was significantly higher in CZP 1 mg/kg group compared to controls. Almost half of CZP-exposed rats stayed for some time close to the connecting hole to the home cage before they entered to it. It means, that they found their way to the home cage but they did not use it immediately. Such deficits can be related to prior knowledge of the box that reduced the tendency to explore and decreased motivation to approach the nest with littermates. Decreased motivation as a consequence of BZs-exposure was shown previously in other experiments (Coen et al., 1983; Fox et al., 1977). Thus, we hypothesize that mild alteration of homing reaction seen in CZP-exposed animals rather due to decreased motivation than impaired memory.

Habituation is considered as an elementary type of non-associative learning manifested as decrease of exploratory activity with repeated exposure to an unfamiliar environment (Platel and Porsolt, 1982). In present study, short-term habituation was defined as decrease of locomotion between two 5-min intervals in the first and forth session. Long-term habituation was defined as a decrease of locomotion in the corresponding 5-min intervals between the first and the fourth testing session. Our data did not show any impairment of short-time habituation. In contrast, long-term habituation was impaired and CZP-exposed animals did not habituate. In the same CZP-exposed animals exhibited increased locomotor activity in the open field. The increased activity of animals exposed to BZs early during development was reported also by other studies. Fox and collaborators (1977) showed that mice treated with

BZs both pre- and postnatally (to P21 or P35) were hyperactive in T-maze. Similarly, consistent and lasting increase of locomotor activity was diagnosed in rats exposed to diazepam via lactation (P3-P18) (Frieder et al., 1984). Finally, increased activity was found in adult rats receiving diazepam from P2 to P21 (Schroeder et al., 1997). Our data suggest that persisting hyperactivity can develop even after much shorter exposure to BZs. We can conclude that deficit of long-term habituation can represent the impairment of non-associative learning, however, changes in locomotor activity can also play important role in this alteration.

During the open field exposure, controls spent relatively more time in thigmotactic zone and they tried to avoid the center zone. Conversely, early CZP-exposed rats evidently tended to spent longer time in the center than controls. Ratio between time spent in the thigmotactic zone and center zone is considered as a rate of anxiety-related behavior in the open-field. Thus, our data suggest that early CZP-exposure results to anxiety reduction. Our data are in accordance with the reduction in anxiety in the open-field after the acute injection of chlordiazepoxide in the amygdala (McNamara and Skelton, 1993), which mediates fear and anxiety (Davis, 1992). Anxiolytic effect of BZs were repeatedly demonstrated by many experimental studies (Sanger, 1985). In experimental studies anxiolytic effects are most frequently assessed using the elevated plus maze test. In this test, administration of BZs was found to increase the number of entries to the open arms and time spent there (Pellow et al., 1985; Pellow and File, 1986). In our study, early CZP-exposure did not significantly alter behavior of rats in EPM when tested as adults compared to controls, even though there was tendency to spend more time on open arms and to visit open arms more frequently than controls. Previous studies demonstrate long-term changes of anxiety in animals exposed to BZS early in the postnatal life. Schroder and collaborators (1997) reported that postnatal diazepam treatment (P2 to P21) reduced anxiety in animals tested 2 month later. In contrast, File (1987) observed that administration of diazepam in P1-P7 rats does not lead to changes of level of anxiety during adolescence (P35-42). Thus, effects of early BZs exposure on anxiety-like behavior later in the life is probably related to the duration of exposure, but age at exposure can also play a role in later consequences. However, the role of this later parameter has to be further analyzed.

The impairment of cognitive functions is considered to be the most important adverse effect. Cognitive deficits can severely influence day-to-day life and separate children from normal society. Thus, in present study several various methods were used to assess risk of cognitive impairment after relatively short CZP-exposure. In EPM, the transfer latency

employed as well-suited method for measurement of learning and memory (Itoh et al., 1990). Early CZP exposure did not affect this parameter in any of tested dose groups. In fact, rats treated with two highest doses of CZP were slightly faster than controls suggesting that increase of locomotor activity observed in CZP-exposed rats can change behavior in this test. Our data thus support results of previously published studies which did not demonstrate any adverse effects of early BZs exposure on cognitive abilities later in life (Fox et al., 1977; Frieder et al., 1984; Schroeder et al., 1997). In addition, early CZP exposure did not affect escape latencies in Morris water maze test. In contrast, early CZP exposure resulted to decrease success rate, i.e. these rats were more frequently unable to find hidden platform. This effect was not dose dependent, but it is possible that early exposure to CZP affects the escape strategy and rats start to search hidden platform later than controls. This may reflect rather changes in motivational or associative processes as reported previously (Coen et al., 1983; Fox et al., 1977) than cognitive impairment.

Changes in social behavior found in adolescent rats exposed to CZP represent the most important result of present study. CZP exposure affected behavior related to play in a dose-dependent manner. Animals receiving two highest doses of CZP spent less time playing and also number of inter-animal contacts during play was lower. Exposure to the highest dose of CZP, 1mg/kg, influenced also behavior interaction unrelated to play. CZP in doses of 0.5 and 1.0 mg/kg altered mainly the investigative components of social interaction what can be related to decreased motivation or to emotional changes described before. There is clear discrepancy between our data and results of previously published studies. File (1986 a, b) did not found any difference in social interactions between controls and animals exposed to CZP (0.5 – 1 mg/kg) between P1 and P21 in this test, whereas animals exposed to other BZs (lorazepam and diazepam) exhibited increased social interaction. This difference can be explained by differences in used methodology of evaluation (different types of behavior were evaluated), but different exposure time can also play a role in this differences. Thus, further studies are necessary to identify effects of early BZ-exposure on social behavior and its development.

7. Conclusions

Present study demonstrates that daily administration of clonazepam (0.1 – 1 mg/kg; CZP) from P7 to P11 has only minimal impact on normal body growth and it also did not increase mortality of experimental animals. On the other hand, we found both short- and long-term functional changes in animals with early BZs experience. BZs exposure altered social play behavior in juvenile rats. In contrast, memory and learning abilities were not affected. Except social interactions, behavioral changes did not exhibit clear dependence on the used dose. In parallel experiments with PTZ, both seizure susceptibility and severity of seizures were significantly higher up to 6 days after the last administration of CZP (1mg/kg). Thus, we expect that even short-term administration of benzodiazepines which does not alter significantly body growth and normal maturation can affect transiently and/or permanently specific brain functions. We hypothesize that functional changes seen in animals exposed to BZS early in the life can be related to the re-modeling of underlying functional circuitries. Data obtained in this thesis will extend common knowledge about risks of early BZs treatment and mechanisms participating in functional changes.

8. References

1. Adlard, B. P., Dobbing, J., and Smart, J. L. An alternative animal model for the full-term small-for-dates human baby. Biol Neonate 23:95-108; 1973.
2. Alleva, E., and Bignami, G. Prenatal benzodiazepine effects in mice: postnatal behavioral development, response to drug challenges, and adult discrimination learning. Neurotoxicology 7:303-17; 1986.
3. Altman, J., and Sudarshan, K. Postnatal development of locomotion in the laboratory rat. Anim Behav 23:896-920; 1975.
4. Barker, J. L., Behar, T., Li, Y. X., Liu, Q. Y., Ma, W., Maric, D., Maric, I., Schaffner, A. E., Serafini, R., Smith, S. V., Somogyi, R., Vautrin, J. Y., Wen, X. L., and Xian, H. GABAergic cells and signals in CNS development. Perspect Dev Neurobiol 5:305-22; 1998.
5. Ben-Ari, Y., Cherubini, E., Corradetti, R., and Gaiarsa, J. L. Giant synaptic potentials in immature rat CA3 hippocampal neurones. J Physiol 416:303-25; 1989.
6. Ben-Ari, Y. Excitatory actions of gaba during development: the nature of the nurture. Nat Rev Neurosci 3:728-39; 2002.
7. Bignami, G. Economical test methods for developmental neurobehavioral toxicity. Environ Health Perspect 104 Suppl 2:285-98; 1996.
8. Bouwknecht, J. A., Spiga, F., Staub, D. R., Hale, M. W., Shekhar, A., and Lowry, C. A. Differential effects of exposure to low-light or high-light open-field on anxiety-related behaviors: relationship to c-Fos expression in serotonergic and non-serotonergic neurons in the dorsal raphe nucleus. Brain Res Bull 72:32-43; 2007.
9. Bowery, N. G. GABAB receptor: a site of therapeutic benefit. Curr Opin Pharmacol 6:37-43; 2006.
10. Brooks-Kayal, A. R., Shumate, M. D., Jin, H., Rikhter, T. Y., Kelly, M. E., and Coulter, D. A. gamma-Aminobutyric acid(A) receptor subunit expression predicts functional changes in hippocampal dentate granule cells during postnatal development. J Neurochem 77:1266-78; 2001.
11. Browne, T. R., and Penry, J. K. Benzodiazepines in the treatment of epilepsy. A review. Epilepsia 14:277-310; 1973.
12. Buffett-Jerrott, S. E., and Stewart, S. H. Cognitive and sedative effects of benzodiazepine use. Curr Pharm Des 8:45-58; 2002.

13. Burghardt, G. M. Play. Comparative psychology: A handbook 725-735; 1999.
14. Calcagnetti, D. J., and Schechter, M. D. Place conditioning reveals the rewarding aspect of social interaction in juvenile rats. Physiol Behav 51:667-72; 1992.
15. Cerbone, A., and Sadile, A. G. Behavioral habituation to spatial novelty: interference and noninterference studies. Neurosci Biobehav Rev 18:497-518; 1994.
16. Chen, S., Huang, X., Zeng, X. J., Sieghart, W., and Tietz, E. I. Benzodiazepine-mediated regulation of alpha1, alpha2, beta1-3 and gamma2 GABA(A) receptor subunit proteins in the rat brain hippocampus and cortex. Neuroscience 93:33-44; 1999.
17. Coen, E., Abbracchio, M. P., Balduini, W., Cagiano, R., Cuomo, V., Lombardelli, G., Peruzzi, G., Ragusa, M. C., and Cattabeni, F. Early postnatal chlordiazepoxide administration: permanent behavioural effects in the mature rat and possible involvement of the GABA-benzodiazepine system. Psychopharmacology (Berl) 81:261-6; 1983.
18. Collis, R., Plaat, F., Urguhart, J. Textbook of Obstetric Anaesthesia. ISBN 1 900151 77 4; 2002
19. Corbett, R., Fielding, S., Cornfeldt, M., and Dunn, R. W. GABAmimetic agents display anxiolytic-like effects in the social interaction and elevated plus maze procedures. Psychopharmacology (Berl) 104:312-6; 1991.
20. Davis, M. The role of the amygdala in fear and anxiety. Annu Rev Neurosci 15:353-75; 1992.
21. Derry, J. M., Dunn, S. M., and Davies, M. Identification of a residue in the gamma-aminobutyric acid type A receptor alpha subunit that differentially affects diazepam-sensitive and -insensitive benzodiazepine site binding. J Neurochem 88:1431-8; 2004.
22. Dobbing, J. The kinetics of growth. Lancet 2:1358; 1970.
23. Dobbing, J. Undernutrition and the developing brain: the use of animal models ot elucidate the human problem. Psychiatr Neurol Neurochir 74:433-42; 1971.
24. Dreyfus Brisac, C., Lezine, I., and Berges, J. [the Development of the Premature Infant after 2 Years. Psychological, Neurologic and Electroencephalographic Interrelations.]. Rev Neuropsychiatr Infant 12:283-334; 1964.
25. Dulac, O. Epilepsy in children. Curr Opin Neurol. 7:102-106; 1994
26. Eberhard-Gran, M., Eskild, A., and Opjordsmoen, S. Treating mood disorders during pregnancy: safety considerations. Drug Saf 28:695-706; 2005.
27. Farrell, K. Benzodiazepines in the treatment of children with epilepsy. Epilepsia 27 Suppl 1:S45-52; 1986.

29. File, S. E. Rapid development of tolerance to the sedative effects of lorazepam and triazolam in rats. Psychopharmacology (Berl) 73:240-5; 1981.
30. File, S. E. Tolerance to the behavioral actions of benzodiazepines. Neurosci Biobehav Rev 9:113-21; 1985.
31. File, S. E. The effects of neonatal administration of clonazepam on passive avoidance and on social, aggressive and exploratory behavior of adolescent male rats. Neurobehav Toxicol Teratol 8:447-52; 1986.
32. File, S. E. Effects of neonatal administration of diazepam and lorazepam on performance of adolescent rats in tests of anxiety, aggression, learning and convulsions. Neurobehav Toxicol Teratol 8:301-6; 1986.
33. File, S. E. Behavioral changes persisting into adulthood after neonatal benzodiazepine administration in the rat. Neurobehav Toxicol Teratol 8:453-61; 1986.
34. File, S. E. Diazepam and caffeine administration during the first week of life: changes in neonatal and adolescent behavior. Neurotoxicol Teratol 9:9-16; 1987.
35. Fox, K. A., Abendschein, D. R., and Lahcen, R. B. Effects of benzodiazepines during gestation and infancy on Y-maze performance of mice. Pharmacol Res Commun 9:325-38; 1977.
36. Frieder, B., Epstein, S., and Grimm, V. E. The effects of exposure to diazepam during various stages of gestation or during lactation on the development and behavior of rat pups. Psychopharmacology (Berl) 83:51-5; 1984.
37. Gai, N., and Grimm, V. E. The effect of prenatal exposure to diazepam on aspects of postnatal development and behavior in rats. Psychopharmacology (Berl) 78:225-9; 1982.
38. Ganguly, K., Schinder, A. F., Wong, S. T., and Poo, M. GABA itself promotes the developmental switch of neuronal GABAergic responses from excitation to inhibition. Cell 105:521-32; 2001.
39. Gardner, C. R. Distress vocalization in rat pups. A simple screening method for anxiolytic drugs. J Pharmacol Methods 14:181-7; 1985.
40. Haefely, W. E., Kyburz, E., Gerecke, M., Mohler, H. Recent advances in the molecular pharmacology of benzodiazepine receptors and in the structure-activity relationships of their agonists and antagonists. Adv Drug Res 14:165-322; 1985.
41. Haefely, W. E. Benzodiazepines. Int Anesthesiol Clin 26:262-72; 1988.
42. Heinonen, O.P., Slone, D., Shapiro, S. Birth Defects and Drugs in Pregnancy. PSG Publishers, Litteleton; 1977.

43. Hutchinson, M. A., Smith, P. F., and Darlington, C. L. The behavioural and neuronal effects of the chronic administration of benzodiazepine anxiolytic and hypnotic drugs. Prog Neurobiol 49:73-97; 1996.
44. Iqbal, M. M., Sobhan, T., and Ryals, T. Effects of commonly used benzodiazepines on the fetus, the neonate, and the nursing infant. Psychiatr Serv 53:39-49; 2002.
45. Itoh, J., Nabeshima, T., and Kameyama, T. Utility of an elevated plus-maze for dissociation of amnesic and behavioral effects of drugs in mice. Eur J Pharmacol 194:71-6; 1991.
46. Johnston, G. A. GABAc receptors: relatively simple transmitter -gated ion channels? Trends Pharmacol Sci 17:319-23; 1996.
47. Kaneko, S. Antiepileptic drug therapy and reproductive consequences: functional and morphologic effects. Reprod Toxicol 5:179-98; 1991.
48. Kapur, J., Haas, K. F., and Macdonald, R. L. Physiological properties of GABAA receptors from acutely dissociated rat dentate granule cells. J Neurophysiol 81:2464-71; 1999.
49. Kubova, H., and Mares, P. Time course of the anticonvulsant action of clonazepam during ontogenesis in the rat. Arch Int Pharmacodyn Ther 298:15-24; 1989.
50. Kubova, H., Mikulecka, A., Haugvicova, R., Mares, P. The benzodiazepine receptor partial agonist Ro 19-8022 suppresses generalized seizures without impairing motor functions in developing rats. Naunyn Schmiedebergs Arch Pharmacol 360:565-574; 1999
51. Kubova, H., Mares, P., Suchomelova, L., Brozek, G., Druga, R., and Pitkanen, A. Status epilepticus in immature rats leads to behavioural and cognitive impairment and epileptogenesis. Eur J Neurosci 19:3255-65; 2004.
52. Laurie, D. J., Wisden, W., and Seeburg, P. H. The distribution of thirteen GABAA receptor subunit mRNAs in the rat brain. III. Embryonic and postnatal development. J Neurosci 12:4151-72; 1992.
53. Laviola, G., Renna, G., Bignami, G., and Cuomo, V. Ontogenetic and pharmacological dissociation of various components of locomotor activity and habituation in the rat. Int J Dev Neurosci 6:431-8; 1988.
54. Lister, R. G. Ethologically-based animal models of anxiety disorders. Pharmacol Ther 46:321-40; 1990.

55. Madsen, K. K., Larsson, O. M., and Schousboe, A. Regulation of excitation by GABA neurotransmission: focus on metabolism and transport. Results Probl Cell Differ 44:201-21; 2008.
56. Marriott, S., and Tyrer, P. Benzodiazepine dependence. Avoidance and withdrawal. Drug Saf 9:93-103; 1993.
57. Mateffyova, A., Otahal, J., Tsenov, G., Mares, P., and Kubova, H. Intrahippocampal injection of endothelin-1 in immature rats results in neuronal death, development of epilepsy and behavioral abnormalities later in life. Eur J Neurosci 24:351-60; 2006.
58. McElhatton, P. R. The effects of benzodiazepine use during pregnancy and lactation. Reprod Toxicol 8:461-75; 1994.
59. McNamara, R. K., and Skeleton, R. W. Effects of intracranial infusions of chlordiazepoxide on spatial learning in the Morris water maze. I. Neuroanatomical specificity. Behav Brain Res 59:175-91; 1993.
60. Meaney, M.J., Stewart, J. Environmental factor influencing the affiliative behavior of male and female rats. Anim Learn Behav 7:397-405; 1979.
61. Meaney, M.J., Stewart, J. A descriptive study of social development in the rat (rattus norvegicus). Anim Behav 29:34-45; 1981.
62. Mikulecka, A., Krsek, P., and Mares, P. Nonconvulsive Kainic Acid-Induced Seizures Elicit Age-Dependent Impairment of Memory for the Elevated Plus-Maze. Epilepsy Behav 1:418-426; 2000.
63. Mikulecka, A., and Mares, P. Effects of mGluR5 and mGluR1 antagonists on anxiety-like behavior and learning in developing rats. Behav Brain Res 204:133-9; 2009.
64. Mizrahi, E. M., and Clancy, R. R. Neonatal seizures: early-onset seizure syndromes and their consequences for development. Ment Retard Dev Disabil Res Rev 6:229-41; 2000.
65. Mohler, H. GABA(A) receptor diversity and pharmacology. Cell Tissue Res 326:505-16; 2006.
66. Mohler, H. Molecular regulation of cognitive functions and developmental plasticity: impact of GABAA receptors. J Neurochem 102:1-12; 2007.
67. Morris, R. Developments of a water-maze procedure for studying spatial learning in the rat. J Neurosci Methods 11:47-60; 1984.
68. Naito, H., Nakamura, A., and Inoue, M. Ontogenetic changes in responsiveness to benzodiazepine receptor ligands on ultrasonic vocalizations in rat pups. Exp Anim 47:89-96; 1998.

69. Nulman, I., Laslo, D., and Koren, G. Treatment of epilepsy in pregnancy. Drugs 57:535-44; 1999.
70. O'Brien C, P. Benzodiazepine use, abuse, and dependence. J Clin Psychiatry 66 Suppl 2:28-33; 2005.
71. Otto, M. W., Bruce, S. E., and Deckersbach, T. Benzodiazepine use, cognitive impairment, and cognitive-behavioral therapy for anxiety disorders: issues in the treatment of a patient in need. J Clin Psychiatry 66 Suppl 2:34-8; 2005.
72. Owens, D. F., and Kriegstein, A. R. Is there more to GABA than synaptic inhibition? Nat Rev Neurosci 3:715-27; 2002.
73. Pellow, S., Chopin, P., File, S. E., and Briley, M. Validation of open:closed arm entries in an elevated plus-maze as a measure of anxiety in the rat. J Neurosci Methods 14:149-67; 1985.
74. Pellow, S., and File, S. E. Anxiolytic and anxiogenic drug effects on exploratory activity in an elevated plus-maze: a novel test of anxiety in the rat. Pharmacol Biochem Behav 24:525-9; 1986.
75. Petroff, O. A. GABA and glutamate in the human brain. Neuroscientist 8:562-73; 2002.
76. Piacsek, B. E., and Goodspeed, M. P. Maturation of the pituitary-gonadal system in the male rat. J Reprod Fertil 52:29-35; 1978.
77. Pirker, S., Schwarzer, C., Wieselthaler, A., Sieghart, W., and Sperk, G. GABA(A) receptors: immunocytochemical distribution of 13 subunits in the adult rat brain. Neuroscience 101:815-50; 2000.
78. Platel, A., and Porsolt, R. D. Habituation of exploratory activity in mice: a screening test for memory enhancing drugs. Psychopharmacology (Berl) 78:346-52; 1982.
79. Pokorny, J., Yamamoto, T. Postnatal ontogenesis of hippocampal CA1 area in rats. I. Development of dendritic arborisation in pyramidal neurons. Brain Res Bull 7:113-120; 1981
80. Raol, Y. H., Zhang, G., Budreck, E. C., and Brooks-Kayal, A. R. Long-term effects of diazepam and phenobarbital treatment during development on GABA receptors, transporters and glutamic acid decarboxylase. Neuroscience 132:399-407; 2005.
81. Riss, J., Cloyd, J., Gates, J., and Collins, S. Benzodiazepines in epilepsy: pharmacology and pharmacokinetics. Acta Neurol Scand 118:69-86; 2008.
82. Rivera, C., Voipio, J., Payne, J. A., Ruusuvuori, E., Lahtinen, H., Lamsa, K., Pirvola, U., Saarma, M., and Kaila, K. The K+/Cl- co-transporter KCC2 renders GABA hyperpolarizing during neuronal maturation. Nature 397:251-5; 1999.

83. Roberts, A. A., Pleger, G. L., and Kellogg, C. K. Effect of prenatal exposure to diazepam on brain GABA(A) receptor mRNA levels in rats examined at late fetal or adult ages. Dev Neurosci 23:135-44; 2001.
84. Rosenbaum, J. F. Attitudes toward benzodiazepines over the years. J Clin Psychiatry 66 Suppl 2:4-8; 2005.
85. Rothe, T., and Bigl, V. The ontogeny of benzodiazepine receptors in selected regions of the rat brain: effect of perinatal exposure to diazepam. Neuropharmacology 28:503-8; 1989.
86. Sanger, D. J. GABA and the behavioral effects of anxiolytic drugs. Life Sci 36:1503-13; 1985.
87. Schenk, F. Development of place navigation in rats from weaning to puberty. Behav Neural Biol 43:69-85; 1985.
88. Schenk, F. A homing procedure for studying spatial memory in immature and adult rodents. J Neurosci Methods 26:249-58; 1989.
89. Schlumpf, M., Ramseier, H., Abriel, H., Youmbi, M., Baumann, J. B., and Lictensteiger, W. Diazepam effects on the fetus. Neurotoxicology 10:501-16; 1989.
90. Schroeder, H., Humbert, A. C., Desor, D., and Nehlig, A. Long-term consequences of neonatal exposure to diazepam on cerebral glucose utilization, learning, memory and anxiety. Brain Res 766:142-52; 1997.
91. Sieghart, W. GABAA receptors: ligand-gated Cl- ion channels modulated by multiple drug-binding sites. Trends Pharmacol Sci 13:446-50; 1992.
92. Sigel, E. Mapping of the benzodiazepine recognition site on GABA(A) receptors. Curr Top Med Chem 2:833-9; 2002.
93. Spear, L. P. Neurobehavioral assessment during the early postnatal period. Neurotoxicol Teratol 12:489-95; 1990.
94. Stevens, J. C., and Pollack, M. H. Benzodiazepines in clinical practice: consideration of their long-term use and alternative agents. J Clin Psychiatry 66 Suppl 2:21-7; 2005.
95. Stewart, S. A. The effects of benzodiazepines on cognition. J Clin Psychiatry 66 Suppl 2:9-13; 2005.
96. Thiel, C. M., Muller, C. P., Huston, J. P., and Schwarting, R. K. High versus low reactivity to a novel environment: behavioural, pharmacological and neurochemical assessments. Neuroscience 93:243-51; 1999.
97. Tietz, E. I., Huang, X., Chen, S., and Ferencak, W. F., 3rd Temporal and regional regulation of alpha1, beta2 and beta3, but not alpha2, alpha4, alpha5, alpha6, beta1 or

gamma2 GABA(A) receptor subunit messenger RNAs following one-week oral flurazepam administration. Neuroscience 91:327-41; 1999.

98. Trezza, V., and Vanderschuren, L. J. Bidirectional cannabinoid modulation of social behavior in adolescent rats. Psychopharmacology (Berl) 197:217-27; 2008.
99. Tucker, J. C. Benzodiazepines and the developing rat: a critical review. Neurosci Biobehav Rev 9:101-11; 1985.
100. Van den Berg, C. L., Pijlman, F. T., Koning, H. A., Diergaarde, L., Van Ree, J. M., and Spruijt, B. M. Isolation changes the incentive value of sucrose and social behaviour in juvenile and adult rats. Behav Brain Res 106:133-42; 1999.
101. Vanderschuren, L. J., Niesink, R. J., Spruijt, B. M., and Van Ree, J. M. Influence of environmental factors on social play behavior of juvenile rats. Physiol Behav 58:119-23; 1995.
102. Werry, J.S., Aman, M.G. Practiotioner's guide to Psychoactive Drugs for Children and Adolescents ISBN 0 306 45885 3; 1999